

## STREPTOCOCCUS ANTIGENS

This application claims the benefit of US provisional application 60/212,683 filed June 20, 2000

5 which is herein incorporated by reference.

### FIELD OF THE INVENTION

10 The present invention is related to antigens, epitopes and antibodies directed to these epitopes, more particularly polypeptide antigens of streptococcus pneumoniae pathogen which may be useful for prophylaxis, diagnostic or treatment of streptococcal infection.

### BACKGROUND OF THE INVENTION

15 S. pneumoniae is an important agent of disease in man especially among infants, the elderly and immunocompromised persons. It is a bacterium frequently isolated from patients with invasive diseases such as bacteraemia/septicaemia, pneumonia, meningitis with high morbidity and mortality throughout the world. Even  
20 with appropriate antibiotic therapy, pneumococcal infections still result in many deaths. Although the advent of antimicrobial drugs has reduced the overall mortality from pneumococcal disease, the presence of resistant pneumococcal  
25 organisms has become a major problem in the world today. Effective pneumococcal vaccines could have a major impact on the morbidity and mortality associated with S. pneumoniae disease. Such vaccines would also potentially be useful to prevent otitis media in infants and young children.

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Efforts to develop a pneumococcal vaccine have generally concentrated on generating immune responses to the pneumococcal capsular polysaccharide. More than 80 pneumococcal capsular serotypes have been identified on the basis of antigenic

differences. The currently available pneumococcal vaccine, comprising 23 capsular polysaccharides that most frequently caused disease, has significant shortcomings related primarily to the poor immunogenicity of some capsular polysaccharides, the diversity of the serotypes and the differences in the distribution of serotypes over time, geographic areas and age groups. In particular, the failure of existing vaccines and capsular conjugate vaccines currently in development to protect young children against all serotypes spurs evaluation of other S. pneumoniae components. Although immunogenicity of capsular polysaccharides can be improved, serotype specificity will still represent a major limitation of polysaccharide-based vaccines. The use of a antigenically conserved immunogenic pneumococcal protein antigen, either by itself or in combination with additional components, offers the possibility of a protein-based pneumococcal vaccine.

PCT WO 98/18930 published May 7, 1998 entitled "*Streptococcus Pneumoniae* antigens and vaccines" describes certain polypeptides which are claimed to be antigenic. However, no biological activity of these polypeptides is reported. Similarly, no sequence conservation is reported, which is a necessary species common vaccine candidate.

PCT WO 00/39299 describes polypeptides and polynucleotides encoding these polypeptides. PCT WO 00/39299 demonstrates that polypeptides designated as BVH-3 and BVH-11 provide protection against fatal experimental infection with pneumococci.

Therefore there remains an unmet need for *Streptococcus* antigens that may be used as components for the prophylaxis, diagnostic and/or therapy of *Streptococcus* infection.

## SUMMARY OF THE INVENTION

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An isolated polynucleotide comprising a polynucleotide chosen from;

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(a) a polynucleotide encoding a polypeptide having at least 70% identity to a second polypeptide chosen from: table A, B, D, E or H;

(b) a polynucleotide encoding a polypeptide having at least 95% identity to a second polypeptide chosen from: table A, B, D, E or H;

(c) a polynucleotide encoding a polypeptide having an amino sequence chosen from table A, B, D, E or H; or fragments, analogs or derivatives thereof;

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(d) a polynucleotide encoding a polypeptide chosen from: table A, B, D, E or H;

(e) a polynucleotide encoding a polypeptide capable of generating antibodies having binding specificity for a polypeptide having a sequence chosen from: table A, B, D, E or H;

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(f) a polynucleotide encoding an epitope bearing portion of a polypeptide chosen from table A, B, D, E or H; and

(g) a polynucleotide complementary to a polynucleotide in (a), (b), (c), (d), (e) or (f).

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In other aspects, there are provided novel polypeptides encoded by polynucleotides of the invention, pharmaceutical or vaccine composition, vectors comprising polynucleotides of the invention operably linked to an expression control region, as well as host cells transfected with said vectors and methods of producing polypeptides comprising culturing said host cells under

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conditions suitable for expression.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is the DNA sequence of SP64 BVH-3 gene; SEQ ID NO: 1

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Figure 2 is a DNA sequence containing the complete SP64 BVH-3 gene at nucleotides 1777 to 4896; SEQ ID NO: 2

Figure 3 is the DNA sequence of SP64 BVH-11 gene; SEQ ID NO: 3

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Figure 4 is a DNA sequence containing the complete SP64 BVH-11 gene at nucleotides 45 to 2567; SEQ ID NO: 4

Figure 5 is a DNA sequence containing the complete SP64 BVH-11-2 gene at nucleotides 114 to 2630; SEQ ID NO: 5

Figure 6 is the amino acid sequence of SP64 BVH-3 polypeptide; SEQ ID NO: 6

Figure 7 is the amino acid sequence of SP64 BVH-11 polypeptide; SEQ ID NO: 7

Figure 8 is the amino acid sequence of SP64 BVH-11-2 polypeptide; SEQ ID NO: 8

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Figure 9 is the DNA sequence of SP63 BVH-3 gene; SEQ ID NO: 9

Figure 10 is the amino acid sequence of SP63 BVH-3 polypeptide; SEQ ID NO: 10

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Figure 11 is the amino acid sequence of 4D4.9 polypeptide; SEQ ID NO: 11

Figure 12 is the amino acid sequence of 7G11.7 polypeptide; SEQ  
ID NO: 12

5 Figure 13 is the amino acid sequence of 7G11.9 polypeptide; SEQ  
ID NO: 13

Figure 14 is the amino acid sequence of 4D3.4 polypeptide; SEQ  
ID NO: 14

10 Figure 15 is the amino acid sequence of 8E3.1 polypeptide; SEQ  
ID NO: 15

Figure 16 is the amino acid sequence of 1G2.2 polypeptide; SEQ  
ID NO: 16

Figure 17 is the amino acid sequence of 10C12.7 polypeptide; SEQ  
ID NO: 17

Figure 18 is the amino acid sequence of 14F6.3 polypeptide; SEQ  
ID NO: 18

Figure 19 is the amino acid sequence of B12D8.2 polypeptide; SEQ  
ID NO: 19

25 Figure 20 is the amino acid sequence of 7F4.1 polypeptide; SEQ  
ID NO: 20

Figure 21 is the amino acid sequence of 10D7.5 polypeptide; SEQ  
ID NO: 21

30 Figure 22 is the amino acid sequence of 10G9.3 polypeptide,  
10A2.2 polypeptide and B11B8.1 polypeptide; SEQ ID NO: 22

Figure 23 is the amino acid sequence of 11B8.4 polypeptide; SEQ ID NO: 23

5 Figure 24 is the amino acid sequence of Mab H11B-11B8 target epitope; SEQ ID 163

10 Figure 25 is a schematic representation of the BVH-3 gene as well as location of gene sequences coding for the full length and truncated polypeptides. The relationships between DNA fragments are shown with respect to each other.

Figure 26 is a schematic representation of the BVH-11 gene as well as location of gene sequences coding for the full length and truncated polypeptides. The relationships between DNA fragments are shown with respect to each other.

20 Figure 27 is a schematic representation of the BVH-11-2 gene as well as location of gene sequences coding for the full length and truncated polypeptides. The relationships between DNA fragments are shown with respect to each other.

25 Figure 28 is a schematic representation of the BVH-3 protein and the location of internal and surface epitopes recognized by certain monoclonal antibodies.

Figure 29 is a schematic representation of the BVH-11-2 protein and the location of protective surface epitopes recognized by certain monoclonal antibodies.

30 Figure 30 is a map of plasmid pURV22.HIS. Kan<sup>R</sup>, kanamycin-resistance coding region; cI857, bacteriophage  $\lambda$  cI857 temperature-sensitive repressor gene; lambda pL, bacteriophage  $\lambda$  transcription promotor; His-tag, 6-histidine coding region;

terminator, T1 transcription terminator; ori, colE1 origin of replication.

Figure 31 depicts the comparison of the amino acid sequences of BVH-3M (sp64) and BVH-3 (Sp63) proteins by using the program Clustal W from MacVector sequence analysis software (version 6.5.3). Underneath the alignment, there is a consensus line where \* and . characters indicate identical and similar amino acid residues, respectively.

Figure 32 depicts the comparison of the amino acid sequences of BVH-3, BVH-11 and BVH-11-2 proteins by using the program Clustal W from MacVector sequence analysis software (version 6.5.3). Underneath the alignment, there is a consensus line where \* and . characters indicate identical and similar amino acid residues, respectively.

Figure 33 is the DNA sequence of the NEW43 gene (SEQ ID No 257).

Figure 34 is the deduced amino acid sequence of NEW43 polypeptide (SEQ ID No 258).

#### DETAILED DESCRIPTION OF THE INVENTION

It was determined that portions of the BVH-3 and BVH-11 polypeptides were internal. Other portions were not present in important strains such as encapsulated s.pneumonia causing disease strains. It would be advantageous to have a polypeptide that comprises a portion that is not internal. When large portions of a polypeptide are internal, these portions are not exposed on the bacteria. However, these portions can be very immunogenic in a recombinant polypeptide and will not confer

protection against infections. It would also be advantageous to have a polypeptide that comprises a portion that is present in most strains.

- 5 The present invention is concerned with polypeptides in which undesired portions have been deleted and/or modified in order to obtain a specific immune response.

10 In accordance with the present invention, there are also provided polypeptides or polynucleotides encoding such polypeptides comprising protective domains.

Surprisingly, when the undesired portion of the polypeptides are deleted or modified, the polypeptides have desired biological properties. This is surprising in view of the fact that some of these portions were described as being epitope bearing portion in the patent application PCT WO 98/18930. In other publications such as PCT WO 00/37105, portions identified as histidine triad and coil coiled regions were said to be of importance. The present inventors have found that variants of the polypeptide BVH-3 and BVH-11 in which certain portions were deleted and/or modified and chimeras of these polypeptides have biological properties and generate a specific immune response.

- 25 According to one aspect, the present invention provides an isolated polynucleotide encoding a polypeptide having at least 70% identity to a second polypeptide comprising a sequence as disclosed in the present application, the tables and figures.

- 30 In accordance with one aspect of the present invention, there is provided an isolated polynucleotide comprising a polynucleotide chosen from;



- (a) a polynucleotide encoding a polypeptide having at least 70% identity to a second polypeptide chosen from: table B, E or H;
- (b) a polynucleotide encoding a polypeptide having at least 95% identity to a second polypeptide chosen from: table B, E or H;
- (c) a polynucleotide encoding a polypeptide having an amino sequence chosen from table B, E or H or fragments, analogs or derivatives thereof;
- (d) a polynucleotide encoding a polypeptide chosen from: table B, E or H;
- (e) a polynucleotide encoding a polypeptide capable of generating antibodies having binding specificity for a polypeptide having a sequence chosen from: table B, E or H,
- (f) a polynucleotide encoding an epitope bearing portion of a polypeptide chosen from table B, E or H; and
- (g) a polynucleotide complementary to a polynucleotide in (a), (b), (c), (d), (e) or (f).

According to one aspect, the present invention provides an isolated polynucleotide encoding a polypeptide having at least 70% identity to a second polypeptide comprising a sequence chosen from table A, B, D, E, G or H or fragments, analogues or derivatives thereof.

According to one aspect, the present invention provides an isolated polynucleotide encoding a polypeptide having at least 95% identity to a second polypeptide comprising a sequence chosen from table A, B, D, E, G or H or fragments, analogues or derivatives thereof.

According to one aspect, the present invention relates to polypeptides characterised by the amino acid sequence chosen

from table A, B, D, E, G or H or fragments, analogues or derivatives thereof.

According to one aspect, the present invention provides an  
5 isolated polynucleotide encoding a polypeptide having at least  
70% identity to a second polypeptide comprising a sequence  
chosen from table A, B, D, E, G or H.

According to one aspect, the present invention provides an  
10 isolated polynucleotide encoding a polypeptide having at least  
95% identity to a second polypeptide comprising a sequence  
chosen from table A, B, D, E, G or H.

According to one aspect, the present invention relates to  
polypeptides characterised by the amino acid sequence chosen  
from table A, B, D, E, G or H.

According to one aspect, the present invention provides an  
isolated polynucleotide encoding a polypeptide having at least  
20 70% identity to a second polypeptide comprising a sequence  
chosen from table B, E or H or fragments, analogues or  
derivatives thereof.

According to one aspect, the present invention provides an  
25 isolated polynucleotide encoding a polypeptide having at least  
95% identity to a second polypeptide comprising a sequence  
chosen from B, E or H or fragments, analogues or derivatives  
thereof.

30 According to one aspect, the present invention relates to  
polypeptides characterised by the amino acid sequence chosen  
from table B, E or H or fragments, analogues or derivatives  
thereof.

According to one aspect, the present invention provides an isolated polynucleotide encoding a polypeptide having at least 70% identity to a second polypeptide comprising a sequence  
5 chosen from table B, E or H.

According to one aspect, the present invention provides an isolated polynucleotide encoding a polypeptide having at least 95% identity to a second polypeptide comprising a sequence  
10 chosen from B, E or H.

According to one aspect, the present invention relates to polypeptides characterised by the amino acid sequence chosen from table B, E or H.

In accordance with the present invention, all nucleotides encoding polypeptides and chimeric polypeptides are within the scope of the present invention.

In a further embodiment, the polypeptides or chimeric polypeptides in accordance with the present invention are antigenic.

In a further embodiment, the polypeptides or chimeric polypeptides in accordance with the present invention are immunogenic.

In a further embodiment, the polypeptides or chimeric polypeptides in accordance with the present invention can elicit an immune response in an individual.

In a further embodiment, the present invention also relates to polypeptides which are able to raise antibodies having binding

specificity to the polypeptides or chimeric polypeptides of the present invention as defined above.

In one embodiment, the polypeptides of table A (BVH-3) or table  
5 D (BVH-11) comprise at least one epitope bearing portion.

In a further embodiment, the fragments of the polypeptides of the present invention will comprise one or more epitope bearing portion identified in Table C and F. The fragment will comprises  
10 at least 15 contiguous amino acid of the polypeptide of table C and F. The fragment will comprises at least 20 contiguous amino acid of the polypeptide of table C and F.

In a further embodiment, the epitope bearing portion of the polypeptide of table A(BVH-3) comprises at least one polypeptide listed in Table C.

In a further embodiment, the epitope bearing portion of the polypeptide of table B(BVH-11) comprises at least one polypeptide listed in Table F.  
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An antibody that " has binding specificity" is an antibody that recognises and binds the selected polypeptide but which does not substantially recognise and bind other molecules in a sample,  
25 such as a biological sample. Specific binding can be measured using an ELISA assay in which the selected polypeptide is used as an antigen.

Unless otherwise defined, all technical and scientific terms  
30 used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in

their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

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10 In accordance with the present invention, "protection" in the biological studies is defined by a significant increase in the survival curve, rate or period. Statistical analysis using the Log rank test to compare survival curves, and Fisher exact test to compare survival rates and numbers of days to death, respectively, might be useful to calculate P values and determine whether the difference between the two groups is statistically significant. P values of 0.05 are regarded as not significant.

15 As used herein, "fragments", "derivatives" or "analogues" of the polypeptides of the invention include those polypeptides in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably conserved) and which may be natural or unnatural. In one embodiment, derivatives and analogues of polypeptides of the invention will have about 70% identity with those sequences illustrated in the figures or fragments thereof. That is, 70% of the residues are the same. In a further embodiment, polypeptides will have greater than 75% homology. In a further embodiment, polypeptides will have greater than 80% homology. In a further embodiment, polypeptides will have greater than 85% homology. In a further embodiment, polypeptides will have greater than 90% homology. In a further embodiment, polypeptides will have greater than 95% homology. In a further embodiment, polypeptides will have greater than 99% homology. In a further embodiment, derivatives and analogues of polypeptides of the invention will have less than about 20 amino acid residue

substitutions, modifications or deletions and more preferably less than 10. Preferred substitutions are those known in the art as conserved i.e. the substituted residues share physical or chemical properties such as hydrophobicity, size, charge or functional groups.

The skilled person will appreciate that analogues or derivatives of the proteins or polypeptides of the invention will also find use in the context of the present invention, i.e. as antigenic/immunogenic material. Thus, for instance proteins or polypeptides which include one or more additions, deletions, substitutions or the like are encompassed by the present invention. In addition, it may be possible to replace one amino acid with another of similar "type". For instance replacing one hydrophobic amino acid with another hydrophilic amino acid.

One can use a program such as the CLUSTAL program to compare amino acid sequences. This program compares amino acid sequences and finds the optimal alignment by inserting spaces in either sequence as appropriate. It is possible to calculate amino acid identity or similarity (identity plus conservation of amino acid type) for an optimal alignment. A program like BLASTx will align the longest stretch of similar sequences and assign a value to the fit. It is thus possible to obtain a comparison where several regions of similarity are found, each having a different score. Both types of identity analysis are contemplated in the present invention.

In an alternative approach, the analogues or derivatives could be fusion proteins, incorporating moieties which render purification easier, for example by effectively tagging the desired protein or polypeptide. It may be necessary to remove

the "tag" or it may be the case that the fusion protein itself retains sufficient antigenicity to be useful.

In an additional aspect of the invention there are provided  
5 antigenic/immunogenic fragments of the proteins or polypeptides of the invention, or of analogues or derivatives thereof.

The fragments of the present invention should include one or more such epitopic regions or be sufficiently similar to such  
10 regions to retain their antigenic/immunogenic properties. Thus, for fragments according to the present invention the degree of identity is perhaps irrelevant, since they may be 100% identical to a particular part of a protein or polypeptide, analogue or derivative as described herein. The key issue, once again, is  
15 that the fragment retains the antigenic/immunogenic properties.

Thus, what is important for analogues, derivatives and fragments is that they possess at least a degree of the antigenicity/immunogenic of the protein or polypeptide from  
20 which they are derived.

In accordance with the present invention, polypeptides of the invention include both polypeptides and chimeric polypeptides.

25 Also included are polypeptides which have fused thereto other compounds which alter the polypeptides biological or pharmacological properties i.e. polyethylene glycol (PEG) to increase half-life; leader or secretory amino acid sequences for ease of purification; prepro- and pro- sequences; and  
30 (poly)saccharides.

Furthermore, in those situations where amino acid regions are found to be polymorphic, it may be desirable to vary one or more

particular amino acids to more effectively mimic the different epitopes of the different streptococcus strains.

Moreover, the polypeptides of the present invention can be modified by terminal  $-NH_2$  acylation (e.g. by acetylation, or thioglycolic acid amidation, terminal carboxy amidation, e.g. with ammonia or methylamine) to provide stability, increased hydrophobicity for linking or binding to a support or other molecule.

Also contemplated are hetero and homo polypeptide multimers of the polypeptide fragments, analogues and derivatives. These polymeric forms include, for example, one or more polypeptides that have been cross-linked with cross-linkers such as avidin/biotin, gluteraldehyde or dimethylsuberimide. Such polymeric forms also include polypeptides containing two or more tandem or inverted contiguous sequences, produced from multicistronic mRNAs generated by recombinant DNA technology.

Preferably, a fragment, analogue or derivative of a polypeptide of the invention will comprise at least one antigenic region i.e. at least one epitope.

In order to achieve the formation of antigenic polymers (i.e. synthetic multimers), polypeptides may be utilised having bishaloacetyl groups, nitroarylhalides, or the like, where the reagents being specific for thio groups. Therefore, the link between two mercapto groups of the different peptides may be a single bond or may be composed of a linking group of at least two, typically at least four, and not more than 16, but usually not more than about 14 carbon atoms.

In a particular embodiment, polypeptide fragments, analogues and



derivatives of the invention do not contain a methionine (Met) starting residue. Preferably, polypeptides will not incorporate a leader or secretory sequence (signal sequence). The signal portion of a polypeptide of the invention may be determined according to established molecular biological techniques. In general, the polypeptide of interest may be isolated from a streptococcus culture and subsequently sequenced to determine the initial residue of the mature protein and therefore the sequence of the mature polypeptide.

According to another aspect, there are provided vaccine compositions comprising one or more streptococcus polypeptides of the invention in admixture with a pharmaceutically acceptable carrier diluent or adjuvant. Suitable adjuvants include oils i.e. Freund's complete or incomplete adjuvant; salts i.e.  $AlK(SO_4)_2$ ,  $AlNa(SO_4)_2$ ,  $AlNH_4(SO_4)_2$ , silica, kaolin, carbon polynucleotides i.e. poly IC and poly AU. Preferred adjuvants include QuilA and Alhydrogel. Vaccines of the invention may be administered parenterally by injection, rapid infusion, nasopharyngeal absorption, dermoabsorption, or bucal or oral. Pharmaceutically acceptable carriers also include tetanus toxoid.

The term vaccine is also meant to include antibodies. In accordance with the present invention, there is also provided the use of one or more antibodies having binding specificity for the polypeptides of the present invention for the treatment or prophylaxis of streptococcus infection and/or diseases and symptoms mediated by streptococcus infection.

Vaccine compositions of the invention are used for the treatment or prophylaxis of streptococcus infection and/or diseases and symptoms mediated by streptococcus infection as described in

P.R. Murray (Ed, in chief), E.J. Baron, M.A. Pfaller, F.C. Tenover and R.H. Yolken. Manual of Clinical Microbiology, ASM Press, Washington, D.C. sixth edition, 1995, 1482p which are herein incorporated by reference. In one embodiment, vaccine compositions of the present invention are used for the treatment or prophylaxis of meningitis, otitis media, bacteremia or pneumonia. In one embodiment, vaccine compositions of the invention are used for the treatment or prophylaxis of streptococcus infection and/or diseases and symptoms mediated by streptococcus infection, in particular S.pneumoniae, group A streptococcus (*pyogenes*), group B streptococcus (GBS or *agalactiae*), *dysgalactiae*, *uberis*, *nocardia* as well as *Staphylococcus aureus*. In a further embodiment, the streptococcus infection is S.pneumoniae.

In a particular embodiment, vaccines are administered to those individuals at risk of streptococcus infection such as infants, elderly and immunocompromised individuals.

As used in the present application, the term " individuals" include mammals. In a further embodiment, the mammal is human.

Vaccine compositions are preferably in unit dosage form of about 0.001 to 100  $\mu\text{g/kg}$  (antigen/body weight) and more preferably 0.01 to 10  $\mu\text{g/kg}$  and most preferably 0.1 to 1  $\mu\text{g/kg}$  1 to 3 times with an interval of about 1 to 6 week intervals between immunizations.

Vaccine compositions are preferably in unit dosage form of about 0.1  $\mu\text{g}$  to 10 mg and more preferably 1 $\mu\text{g}$  to 1 mg and most preferably 10 to 100  $\mu\text{g}$  1 to 3 times with an interval of about 1 to 6 week intervals between immunizations.

According to another aspect, there are provided polynucleotides encoding polypeptides characterised by the amino acid sequence chosen from table A, B, D, E, G or H or fragments, analogues or derivatives thereof.

According to another aspect, there are provided polynucleotides encoding polypeptides characterised by the amino acid sequence chosen from table B, E or H or fragments, analogues or derivatives thereof.

In one embodiment, polynucleotides are those illustrated in table A, B, D, E, G or H which encodes polypeptides of the invention.

In one embodiment, polynucleotides are those illustrated in table B, E or H which encodes polypeptides of the invention.

It will be appreciated that the polynucleotide sequences illustrated in the figures may be altered with degenerate codons yet still encode the polypeptides of the invention. Accordingly the present invention further provides polynucleotides which hybridise to the polynucleotide sequences herein above described (or the complement sequences thereof) having 50% identity between sequences. In one embodiment, at least 70% identity between sequences. In one embodiment, at least 75% identity between sequences. In one embodiment, at least 80% identity between sequences. In one embodiment, at least 85% identity between sequences. In one embodiment, at least 90% identity between sequences. In a further embodiment, polynucleotides are hybridizable under stringent conditions i.e. having at least 95% identity. In a further embodiment, more than 97% identity.

Suitable stringent conditions for hybridation can be readily determined by one of skilled in the art (see for example Sambrook et al., (1989) Molecular cloning : A Laboratory Manual, 2<sup>nd</sup> ed, Cold Spring Harbor, N.Y.; Current Protocols in Molecular Biology, (1999) Edited by Ausubel F.M. et al., John Wiley & Sons, Inc., N.Y.).

In a further embodiment, the present invention provides polynucleotides that hybridise under stringent conditions to either

- (a) a DNA sequence encoding a polypeptide or
- (b) the complement of a DNA sequence encoding a polypeptide;

wherein said polypeptide comprising a sequence chosen from table A, B, D, E, G or H or fragments or analogues thereof.

In a further embodiment, the present invention provides polynucleotides that hybridise under stringent conditions to either

- (c) a DNA sequence encoding a polypeptide or
- (d) the complement of a DNA sequence encoding a polypeptide;

wherein said polypeptide comprising a sequence chosen from table B, E or H or fragments or analogues thereof.

In a further embodiment, the present invention provides polynucleotides that hybridise under stringent conditions to either

- (a) a DNA sequence encoding a polypeptide or
- (b) the complement of a DNA sequence encoding a polypeptide;

wherein said polypeptide comprises at least 10 contiguous amino acid residues from a polypeptide comprising a sequence chosen from table A, B, D, E, G or H or fragments or analogues thereof.

5 In a further embodiment, the present invention provides polynucleotides that hybridise under stringent conditions to either

(c) a DNA sequence encoding a polypeptide or

(d) the complement of a DNA sequence encoding a

10 polypeptide;

wherein said polypeptide comprises at least 10 contiguous amino acid residues from a polypeptide comprising a sequence chosen from table B, E or H or fragments or analogues thereof.

15 In a further embodiment, polynucleotides are those encoding polypeptides of the invention illustrated in table A, B, D, E, G or H.

20 As will be readily appreciated by one skilled in the art, polynucleotides include both DNA and RNA.

25 The present invention also includes polynucleotides complementary to the polynucleotides described in the present application.

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In a further aspect, polynucleotides encoding polypeptides of the invention, or fragments, analogues or derivatives thereof, may be used in a DNA immunization method. That is, they can be incorporated into a vector which is replicable and expressible upon injection thereby producing the antigenic polypeptide in vivo. For example polynucleotides may be incorporated into a plasmid vector under the control of the CMV promoter which is functional in eukaryotic cells. Preferably the vector is

injected intramuscularly.

According to another aspect, there is provided a process for producing polypeptides of the invention by recombinant techniques by expressing a polynucleotide encoding said polypeptide in a host cell and recovering the expressed polypeptide product. Alternatively, the polypeptides can be produced according to established synthetic chemical techniques i.e. solution phase or solid phase synthesis of oligopeptides which are ligated to produce the full polypeptide (block ligation).

General methods for obtention and evaluation of polynucleotides and polypeptides are described in the following references: Sambrook et al, Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> ed, Cold Spring Harbor, N.Y., 1989; Current Protocols in Molecular Biology, Edited by Ausubel F.M. et al., John Wiley and Sons, Inc. New York; PCR Cloning Protocols, from Molecular Cloning to Genetic Engineering, Edited by White B.A., Humana Press, Totowa, New Jersey, 1997, 490 pages; Protein Purification, Principles and Practices, Scopes R.K., Springer-Verlag, New York, 3<sup>rd</sup> Edition, 1993, 380 pages; Current Protocols in Immunology, Edited by Coligan J.E. et al., John Wiley & Sons Inc., New York which are herein incorporated by reference.

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For recombinant production, host cells are transfected with vectors which encode the polypeptide, and then cultured in a nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes. Suitable vectors are those that are viable and replicable in the chosen host and include chromosomal, non-chromosomal and synthetic DNA sequences e.g. bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and

phage DNA. The polypeptide sequence may be incorporated in the vector at the appropriate site using restriction enzymes such that it is operably linked to an expression control region comprising a promoter, ribosome binding site (consensus region or Shine-Dalgarno sequence), and optionally an operator (control element). One can select individual components of the expression control region that are appropriate for a given host and vector according to established molecular biology principles (Sambrook et al, Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> ed, Cold Spring Harbor, N.Y., 1989; Current Protocols in Molecular Biology, Edited by Ausubel F.M. et al., John Wiley and Sons, Inc. New York incorporated herein by reference). Suitable promoters include but are not limited to LTR or SV40 promoter, E.coli lac, tac or trp promoters and the phage lambda P<sub>L</sub> promoter. Vectors will preferably incorporate an origin of replication as well as selection markers i.e. ampicillin resistance gene. Suitable bacterial vectors include pET, pQE70, pQE60, pQE-9, pbs, pD10 phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 and eukaryotic vectors pBlueBacIII, pWLNEO, pSV2CAT, pOG44, pXT1, pSG, pSVK3, pBPV, pMSG and pSVL. Host cells may be bacterial i.e. E.coli, Bacillus subtilis, Streptomyces; fungal i.e. Aspergillus niger, Aspergillus nidulins; yeast i.e. Saccharomyces or eukaryotic i.e. CHO, COS.

Upon expression of the polypeptide in culture, cells are typically harvested by centrifugation then disrupted by physical or chemical means (if the expressed polypeptide is not secreted into the media) and the resulting crude extract retained to isolate the polypeptide of interest. Purification of the polypeptide from culture media or lysate may be achieved by established techniques depending on the properties of the polypeptide i.e. using ammonium sulfate or ethanol precipitation

, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, hydroxylapatite chromatography and lectin chromatography. Final purification may be achieved using HPLC.

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The polypeptide may be expressed with or without a leader or secretion sequence. In the former case the leader may be removed using post-translational processing (see US 4,431,739; US 4,425,437; and US 4,338,397 incorporated herein by reference) or be chemically removed subsequent to purifying the expressed polypeptide.

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According to a further aspect, the streptococcus polypeptides of the invention may be used in a diagnostic test for streptococcus infection, in particular S. pneumoniae infection. Several diagnostic methods are possible, for example detecting streptococcus organism in a biological sample, the following procedure may be followed:

- a) obtaining a biological sample from a patient;
- b) incubating an antibody or fragment thereof reactive with a streptococcus polypeptide of the invention with the biological sample to form a mixture; and
- c) detecting specifically bound antibody or bound fragment in the mixture which indicates the presence of streptococcus.

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Alternatively, a method for the detection of antibody specific to a streptococcus antigen in a biological sample containing or suspected of containing said antibody may be performed as follows:

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- a) obtaining a biological sample from a patient;
- b) incubating one or more streptococcus polypeptides of the invention or fragments thereof with the biological sample to form a mixture; and



c)detecting specifically bound antigen or bound fragment in the mixture which indicates the presence of antibody specific to streptococcus.

5 One of skill in the art will recognize that this diagnostic test may take several forms, including an immunological test such as an enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay or a latex agglutination assay, essentially to determine whether antibodies specific for the polypeptide are present in an  
10 organism.

The DNA sequences encoding polypeptides of the invention may also be used to design DNA probes for use in detecting the presence of streptococcus in a biological sample suspected of containing such bacteria. The detection method of this invention comprises:

- a)obtaining the biological sample from a patient;  
b)incubating one or more DNA probes having a DNA sequence encoding a polypeptide of the invention or fragments thereof with the biological sample to form a mixture; and  
c)detecting specifically bound DNA probe in the mixture which indicates the presence of streptococcus bacteria.

The DNA probes of this invention may also be used for detecting  
25 circulating streptococcus i.e. S.pneumoniae nucleic acids in a sample, for example using a polymerase chain reaction, as a method of diagnosing streptococcus infections. The probe may be synthesized using conventional techniques and may be immobilized on a solid phase, or may be labelled with a detectable label. A  
30 preferred DNA probe for this application is an oligomer having a sequence complementary to at least about 6 contiguous nucleotides of the streptococcus pneumoniae polypeptides of the invention.

Another diagnostic method for the detection of streptococcus in a patient comprises:

- a) labelling an antibody reactive with a polypeptide of the invention or fragment thereof with a detectable label;
- b) administering the labelled antibody or labelled fragment to the patient; and
- c) detecting specifically bound labelled antibody or labelled fragment in the patient which indicates the presence of streptococcus.

A further aspect of the invention is the use of the streptococcus polypeptides of the invention as immunogens for the production of specific antibodies for the diagnosis and in particular the treatment of streptococcus infection. Suitable antibodies may be determined using appropriate screening methods, for example by measuring the ability of a particular antibody to passively protect against streptococcus infection in a test model. One example of an animal model is the mouse model described in the examples herein. The antibody may be a whole antibody or an antigen-binding fragment thereof and may belong to any immunoglobulin class. The antibody or fragment may be of animal origin, specifically of mammalian origin and more specifically of murine, rat or human origin. It may be a natural antibody or a fragment thereof, or if desired, a recombinant antibody or antibody fragment. The term recombinant antibody or antibody fragment means antibody or antibody fragment which was produced using molecular biology techniques. The antibody or antibody fragments may be polyclonal, or preferably monoclonal. It may be specific for a number of epitopes associated with the streptococcus pneumoniae polypeptides but is preferably specific for one.

A further aspect of the invention is the use of the antibodies directed to the streptococcus polypeptides of the invention for passive immunization. One could use the antibodies described in the present application. Suitable antibodies may be determined using appropriate screening methods, for example by measuring the ability of a particular antibody to passively protect against streptococcus infection in a test model. One example of an animal model is the mouse model described in the examples herein. The antibody may be a whole antibody or an antigen-binding fragment thereof and may belong to any immunoglobulin class. The antibody or fragment may be of animal origin, specifically of mammalian origin and more specifically of murine, rat or human origin. It may be a natural antibody or a fragment thereof, or if desired, a recombinant antibody or antibody fragment. The term recombinant antibody or antibody fragment means antibody or antibody fragment which was produced using molecular biology techniques. The antibody or antibody fragments may be polyclonal, or preferably monoclonal. It may be specific for a number of epitopes associated with the streptococcus pneumoniae polypeptides but is preferably specific for one.

The following are reference tables summarizing the sequences disclosed in the present application:

**TABLE A, B and C Variants and Epitope of BVH-3-**

**Table A**

<b><u>Family</u></b>	<b><u>Polypeptide SEQ ID NO</u></b>
BVH-3	
New 21	aa 396-1039 of SEQ ID. 6
New 25	aa 233-1039 of SEQ ID.6
New 40	aa 408-1039 of SEQ ID.6

2WS  
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TABLE B -

<u>Family</u>	<u>Polypeptide SEQ ID NO</u>
BVH-3	
NEW1-mut1**	235
NEW35A	236
NEW42	237
NEW49	238
NEW50	239
NEW51	240
NEW52	241
NEW53	242
NEW54	243
NEW55	244
NEW56	245
NEW56-mut2**	245
NEW56-mut3**	245
NEW57	246
NEW63	247
NEW64	248
NEW65	249
NEW66	250
NEW76	251
NEW105	252
NEW106	253
NEW107	254

- 5 \*\* silent mutation, i.e. the polypeptide is the same as New1 or New 56

TABLE C- Epitopes of BVH-3

7G11.7	12
7G11.9	13
B12D8.2	19
7F4.1	20
14F6.3	18

4D3.4	14
10C12.7	17
8E3.1	15
1G2.2	16

**TABLE D, E and F Variants and Epitope of BVH-11-**

5 **TABLE D-**

<u>Family</u>	<u>Polypeptide SEQ ID NO</u>
BVH-11	
New19	aa 497-838 of Seq.ID 8
New24	aa 227-838 of Seq.ID 8

**TABLE E-**

<u>Family</u>	<u>Polypeptide SEQ ID NO</u>
BVH-11	
New 43	258
NEW60	293
NEW61	294
NEW62	295
NEW80	296
NEW81	297
NEW82	298
NEW83	299
NEW84	300
NEW85	301
NEW88D1	302
NEW88D2	303
NEW88	304

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**TABLE F- epitopes of BVH-11**

10D7.5	21
10G9.3	22
B11B8.1	22
10A2.2	22
11b8.4	23

TABLE G and H Chimeras-TABLE G

Family	Polypeptide SEQ ID NO
Chimeras with BVH-11 and BVH-3	
New17	M*-NEW5-G*P*-NEW1
New20	M*-NEW1-G*P*-NEW5
New26	M*-NEW10-G*P*-NEW25
New27	M*-NEW19-G*P*-NEW25
New28	M*-NEW10-G*P*-NEW1
New29	M*-NEW5-G*P*-NEW25
New30	M*-NEW4-G*P*-NEW25
New31	M*-NEW4-G*P*-NEW1
NEW32	M*-NE19-G*P*-NEW1

\* OPTIONAL AMINO ACID

TABLE H

Family	Polypeptide SEQ ID NO
Chimeras with BVH-11 and BVH-3	
VP 89	305
VP 90	306
VP 91	307
VP 92	308
VP 93	309
VP 94	310
VP 108	311
VP109	312
VP 110	313
VP 111	314
VP112	315
VP113	316
VP114	317
VP115	318
VP116	319
VP117	320
VP119	321
VP120	322
VP121	323
VP122	324

Family	Polypeptide SEQ ID NO
Chimeras with BVH-11 and BVH-3	
VP123	325
VP124	326

#### EXAMPLE 1

This example describes the bacterial strains, plasmids, PCR primers, recombinant proteins and hybridoma antibodies used herein.

S. pneumoniae SP64 (serogroup 6) and SP63 (serogroup 9) clinical isolates were provided by the Laboratoire de la Santé Publique du Québec, Sainte-Anne-de-Bellevue; Rx1 strain, a nonencapsulated derivative of the type 2 strain D39 and the type 3 strain WU2 were provided by David E. Briles from University of Alabama, Birmingham and the type 3 clinical isolate P4241 was provided by the Centre de Recherche en Infectiologie du Centre Hospitalier de l'Université Laval, Sainte-Foy. E. coli strains DH5 $\alpha$  (Gibco BRL, Gaithersburg, MD); AD494 ( $\lambda$ DE3) (Novagen, Madison, WI) and BL21 ( $\lambda$ DE3) (Novagen) as well as plasmid superlinker pSL301 vector (Invitrogen, San Diego, CA); pCMV-GH vector (gift from Dr. Stephen A. Johnston, Department for Biochemistry, University of Texas, Dallas, Texas); pET32 and pET21 (Novagen) and pURV22.HIS expression vectors (Figure 30) were used in this study. The pURV22.HIS vector contains a cassette of the bacteriophage  $\lambda$  cI857 temperature-sensitive repressor gene from which the functional P<sub>R</sub> promoter has been deleted. The inactivation of the cI857 repressor by a temperature increase from the range of 30-37°C to 37-42°C results in the induction of the gene under the control of promoter  $\lambda$ PL. The PCR primers used for the generation of the recombinant plasmids had a restriction endonuclease site at the

5'end, thereby allowing directional cloning of the amplified product into the digested plasmid vector. The PCR oligonucleotide primers used are listed in the following Table 1. The location of the gene sequences coding for BVH-3, BVH-11 and BVH-11-2 gene products is summarized in the Figure 25, Figure 26 and Figure 27, respectively.

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Table 1. List of PCR oligonucleotide primers

Primer	SEQ ID NO	Sequence 5' - 3'	Nucleotide position	Restriction sites
OCRR 479	25	cagtagatctgtgcctatgcact aaac	SEQ ID 1: 61-78 SEQ ID 9: 1-18	BglII
OCRR 480	26	gatctctagactactgctattcc ttacgctatg	SEQ ID 2: 4909-4887 SEQ ID 9: 2528-2519	XbaI
OCRR 497	27	atcactcgagcattacctggata atcctgt	SEQ ID 1: 1525-1506	XhoI
OCRR 498	28	ctgctaagcttatgaaagattta gat	SEQ ID 1: 1534-1548	HindIII
OCRR 499	29	gatactcgagctgctattcctta c	SEQ ID 2: 4906-4893	XhoI
HAMJ 172	30	gaatctcgagttaagctgctgct aattc	SEQ ID 1: 675-661	XhoI
HAMJ 247	31	gacgctcgagcgctatgaaatca gataaattc	SEQ ID 1: 3117-3096	XhoI
HAMJ 248	32	gacgctcgagggcattacctgga taatcctgttcag	SEQ ID 1: 1527-1501	XhoI
HAMJ 249	33	cagtagatctcttcatcatttat tgaaaagagg	SEQ ID 2: 1749-1771	BglII
HAMJ 278	34	ttatttcttccatatggacttga cagaagagcaaattaag	SEQ ID 1: 1414-1437	NdeI
HAMJ 279	35	cgccaagcttcgctatgaaatca gataaattc	SEQ ID 1: 3117-3096	HindIII
HAMJ 280	36	cgccaagcttttccacaataataa gtcgattgatt	SEQ ID 1: 2400-2377	HindIII

Primer	SEQ ID NO	Sequence 5' - 3'	Nucleotide position	Restriction sites
HAMJ 281	37	ttattttcttccatatggaagtac ctatcttggaanaagaa	SEQ ID 1: 2398-2421	NdeI
HAMJ 300	38	ttattttcttccatatggtgccta tgactaaaccagc	SEQ ID 1: 62-82	NdeI
HAMJ 313	39	ataagaatgcggccgcttccaca atataagtcgattgatt	SEQ ID 1: 2400-2377	NotI
OCRR 487	40	cagtagatctgtgcttatgaact aggtttgc	SEQ ID 3: 58-79	BglII
OCRR 488	41	gatcaagcttgctgctaccttta cttactctc	SEQ ID 4: 2577-2556	HindIII
HAMJ 171	42	ctgagatatccgttatcggtcaa acc	SEQ ID 3: 1060-1075	EcoRV
HAMJ 251	43	ctgcaagcttttaaaaggggaata atacg	SEQ ID 3: 1059-1045	HindIII
HAMJ 264	44	cagtagatctgcagaagccttcc tatctg	SEQ ID 3: 682-700	BglII
HAMJ 282	45	tcgccaagcttcggttatcggtca aaccattggg	SEQ ID 3: 1060-1081	HindIII
HAMJ 283	46	ataagaatgcggccgccttactc tcctttaataaaagccaatagtt	SEQ ID 3: 2520-2492	NotI
HAMJ 284	47	catgccatggacattgatagtct cttgaaacagc	SEQ ID 3: 856-880	NcoI
HAMJ 285	48	cgccaagcttcttactctccttt aataaagccaatag	SEQ ID 3: 2520-2494	HindIII
HAMJ 286	49	cgacaagcttaacatggtcgcta gcgttacc	SEQ ID 3: 2139-2119 SEQ ID 5: 2210-2190	HindIII
HAMJ 287	50	cataccatgggcctttatgaggc acctaag	SEQ ID 3: 2014-2034	NcoI

Primer	SEQ ID NO	Sequence 5' - 3'	Nucleotide position	Restriction sites
HAMJ 288	51	cgacaagcttaagtaaattcttca gcctctctcag	SEQ ID 3: 2376-2353	HindIII
HAMJ 289	52	gataccatggctagcgacatgt tcaaagaa	SEQ ID 3: 2125-2146	NcoI
HAMJ 290	53	cgccaagcttatcatccactaac ttgacttttatcac	SEQ ID 3: 1533-1508	HindIII
HAMJ 291	54	cataccatggatattcttgcctt cttagctccg	SEQ ID 3: 1531-1554	NcoI
HAMJ 301	55	catgccatggtgcttatgaacta ggtttgc	SEQ ID 3: 59-79	NcoI
HAMJ 302	56	cgccaagcttttagcgttaccaa accattatc	SEQ ID 3: 2128-2107	HindIII
HAMJ 160	57	gtattagatctgttcctatgaac ttggtcgctcacca	SEQ ID 5: 172-196	BglII
HAMJ 186	58	cgctctagactactgtatagga gccgg	SEQ ID 5: 2613-2630	XbaI
HAMJ 292	59	catgccatggaaaacatttcaag ccttttacgtg	SEQ ID 5: 925-948	NcoI
HAMJ 293	60	cgacaagcttctgtataggagcc ggttgactttc	SEQ ID 5: 2627-2604	HindIII
HAMJ 294	61	catgccatggttcgtaaaaataa ggcagaccaag	SEQ ID 5: 2209-2232	NcoI
HAMJ 297	62	catgccatggaagcctattggaa tggaag	SEQ ID 5: 793-812	NcoI
HAMJ 352	63	catgccatggaagcctattggaa tggaagc	SEQ ID 5: 793-813	NcoI
HAMJ 353	64	cgccaagcttgtaggaatttgc gcatttgg	SEQ ID 5: 1673-1653	HindIII
HAMJ 354	65	cgccaagcttctgtataggagcc ggttgac	SEQ ID 5: 2627-2608	HindIII

Primer	SEQ ID NO	Sequence 5' - 3'	Nucleotide position	Restriction sites
HAMJ 355	66	catgccatggatattcttgcctt cttagctcc	SEQ ID 5: 1603-1624	NcoI
HAMJ 404	67	ttattttcttccatcatgcatgggtg atcatttccattaca	SEQ ID 1: 1186-1207	NdeI
HAMJ 464	68	gatgcatatgaatatgcaaccga gtcagttaagc	SEQ ID 1: 697-720	NdeI
HAMJ 465	69	gatgctcgagagcatcaaattccg tatccatc	SEQ ID 1: 1338-1318	XhoI
HAMJ 466	70	gatgcatatggatcatttccatt acattcca	SEQ ID 1: 1192-1212	NdeI
HAMJ 467	71	gacaagcttggcattacctggat aatcctg	SEQ ID 1: 1527-1507	HindIII
HAMJ 352	72	catgccatggaagcctatttgaa tggaagc	SEQ ID 5: 793-813	NcoI
HAMJ 470	73	ataagaatgcggccgcccgtatg aatcagataaattc	SEQ ID 1: 3096-3117	NotI
HAMJ 471	168	atatggggccctgtataggagcc ggttgactttc	SEQ ID 5: 2626-2604	Apa I
HAMJ 472	169	atatggggcccaatatgcaaccga gtcagttaagc	SEQ ID 1: 720-697	Apa I
HAMJ 350	170	atatggggcccaacatggtcgcta gcgttacc	SEQ ID 3: 2139-2119	Apa I
HAMJ 351	171	tcccggggcccgacttgacagaag agcaaattaag	SEQ ID 1: 1414-1437	Apa I
HAMJ 358	172	catgccatgggacttgacagaag agcaaattaag	SEQ ID 1: 1415-1437	NcoI
HAMJ 359	173	tcccggggcccgctatgaaatca gataaattc	SEQ ID 1: 3116-3096	Apa I
HAMJ 403	174	atatggggcccgacattgatagtc tcttgaaacagc	SEQ ID 3: 856-880	Apa I
HAMJ 361	175	cgccaagcttaacatggtcgcta gcgttacc	SEQ ID 3: 2139-2119	Hind III
HAMJ 483	176	atatggggccccttactctccttt aataaagccaatag	SEQ ID 3: 2520-2494	Apa I

Molecular biology techniques were performed according to standard methods. See for example, Sambrook, J., Fritsch, E.F., Maniatis, T., "Molecular cloning. A laboratory manual" Vol.1-2-3 (second edition) Cold Spring Harbour Laboratory Press, 1989, New York, which is herein incorporated by reference. PCR-amplified products were digested with restriction endonucleases and ligated to either linearized plasmid pSL301, pCMV-GH, pET or pURV22.HIS expression vector digested likewise or digested with enzymes that produce compatible cohesive ends. Recombinant pSL301 and recombinant pCMV-GH plasmids were digested with restriction enzymes for the in-frame cloning in pET expression vector. When pET vectors were used, clones were first stabilized in E. coli DH5 $\alpha$  before introduction into E. coli BL21( $\lambda$ DE3) or AD494 ( $\lambda$ DE3) for expression of full-length or truncated BVH-3, BVH-11 or BVH-11-2 molecules. Each of the resultant plasmid constructs was confirmed by nucleotide sequence analysis. The recombinant proteins were expressed as N-terminal fusions with the thioredoxin and His-tag (pET32 expression system); as C-terminal fusions with an His-tag (pET21 expression system); or as N-terminal fusions with an His-tag (pURV22.HIS expression system). The expressed recombinant proteins were purified from supernatant fractions obtained after centrifugation of sonicated IPTG- (pET systems) or heat- (pURV22.HIS) induced E. coli using a His-Bind metal chelation resin (QIAgen, Chatsworth, CA). The gene products generated from S. pneumoniae SP64 are listed in the following Table 2. The gene fragment encoding BVH-3-Sp63 protein (amino acid residues 21 to 840 on SEQ ID NO: 10) was generated from S. pneumoniae SP63 using the PCR-primer sets OCRR479-OCRR480 and the cloning vector pSL301. The recombinant pSL301-BVH-3Sp63 was digested for the in-frame cloning in pET32 vector for the expression of the BVH-3-Sp63 molecule.

Table 2. Lists of truncated BVH-3, BVH-11, BVH-11-2 and Chimeric gene products generated from *S. pneumoniae* SP64

PCR-primer sets	Protein designation	Identification	Encoded amino acids (SEQ ID No6)	Cloning vector
OCRR479-OCRR480	BVH-	BVH-3 w/o ss	21-1039	pSL301
OCRR479-OCRR497	BVH-	BVH-3 N'end	21-509	pSL301
HAMJ248-HAMJ249	L-BVH-	BVH-3 N'end	1-509	pET-21(+)
OCRR498-OCRR499	BVH-	BVH-3 C'end	512-1039	pSL301
OCRR479-HAMJ172	BVH-	BVH-3 N'end	21-225	pET-32 c(+)
OCRR487-OCRR488	BVH-	BVH-11 w/o ss	20-840	pCMV-GH
HAMJ251-OCRR487	BVH-	BVH-11 N'end	20-353	pET-32 c(+)
HAMJ171-OCRR488	BVH-	BVH-11 C'end	354-840	pET-32 a(+)
HAMJ264-OCRR488	BVH-	BVH-11 C'end	228-840	pET-32 a(+)
HAMJ278-HAMJ279	NEW1	BVH-3 C'end	472-1039	pET-21b(+)
HAMJ278-HAMJ280	NEW2	BVH-3 C'end	472-800	pET-21b(+)
HAMJ281-HAMJ279	NEW3	BVH-3 C'end	800-1039	pET-21b(+)
HAMJ284-HAMJ285	NEW4	BVH-11 C'end	286-840	pET-21d(+)
HAMJ284-HAMJ286	NEW5	BVH-11	286-713	pET-21d(+)
HAMJ287-HAMJ288	NEW6	BVH-11	672-792	pET-21d(+)
HAMJ285-HAMJ289	NEW7	BVH-11 C'end	709-840	pET-21d(+)
HAMJ284-HAMJ290	NEW8	BVH-11	286-511	pET-21d(+)
HAMJ286-HAMJ291	NEW9	BVH-11	511-713	pET-21d(+)
HAMJ160-HAMJ186	BVH-	BVH-11-2 w/o	20-838	pSL301
HAMJ292-HAMJ293	NEW10	BVH-11-2	271-838	pET-21d(+)
HAMJ293-HAMJ294	NEW11	BVH-11-2	699-838	pET-21d(+)
HAMJ282-HAMJ283	NEW13	BVH-11 C'end	354-840	pET-21b(+)
HAMJ286-HAMJ297	NEW14	BVH-11-2	227-699	pET-21d(+)
HAMJ300-HAMJ313	NEW15	BVH-3 N'end	21-800	pET-21b(+)
HAMJ301-HAMJ302	NEW16	BVH-11 N'end w/o ss	20-709	pET-21d(+)
HAMJ352-HAMJ353	NEW18	BVH-11-2 internal	227-520	pET21d(+)

HAMJ354-HAMJ355	NEW19	BVH-11-2 C'end	497-838	pET21d(+)
HAMJ404-HAMJ279	NEW21	BVH-3 C'end	396-1039	pET21b(+)
HAMJ464-HAMJ465	NEW22	BVH-3 internal	233-446	pET-21a(+)
HAMJ466-HAMJ467	NEW23	BVH-3 internal	398-509	pET-21b(+)
HAMJ352-HAMJ293	NEW24	BVH-11-2 C'end	227-838	pET-21d(+)
HAMJ464-HAMJ470	NEW25	BVH-3 C'end	233-1039	pET-21b(+)
HAMJ278-HAMJ279 (NEW 1) HAMJ282- HAMJ283 (NEW 13)	NEW1 2	Chimera*	M-NEW 1 -KL - NEW 13	pET 21 b (+)
HAMJ284-HAMJ350 (NEW 5) HAMJ351- HAMJ279 (NEW 1)	NEW1 7	Chimera*	M- NEW 5 -GP - NEW 1	pET 21 d (+)
HAMJ358-HAMJ359 (NEW 1) HAMJ403- HAMJ361 (NEW 5)	NEW2 0	Chimera*	M- NEW 1 -GP - NEW 5	pET 21 d (+)
HAMJ292-HAMJ471 (NEW 10) HAMJ472- HAMJ470 (NEW 25)	NEW2 6	Chimera*	M- NEW 10 -GP - NEW 25	pET 21 d (+)
HAMJ355-HAMJ471 (NEW 19) HAMJ472- HAMJ470 (NEW 25)	NEW2 7	Chimera*	M- NEW 19 -GP - NEW 25	pET 21 d (+)
HAMJ292-HAMJ471 (NEW 10) HAMJ351 -HAMJ279 (NEW 1)	NEW2 8	Chimera*	M- NEW 10 -GP - NEW 1	pET 21 d (+)
HAMJ284-HAMJ350 (NEW 5) HAMJ472- HAMJ470 (NEW 25)	NEW2 9	Chimera*	M- NEW 5 -GP - NEW 25	pET 21 d (+)
HAMJ284-HAMJ483 (NEW 4) HAMJ472- HAMJ470 (NEW 25)	NEW3 0	Chimera*	M- NEW 4 -GP - NEW 25	pET 21 d (+)
HAMJ284-HAMJ483 (NEW 4) HAMJ351- HAMJ279 (NEW 1)	NEW3 1	Chimera*	M- NEW 4 -GP - NEW 1	pET 21 d (+)
HAMJ355-HAMJ471 (NEW 19) HAMJ351- HAMJ279 (NEW 1)	NEW3 2	Chimera*	M- NEW 19 -GP - NEW 1	pET 21 d (+)

w/o ss : without signal sequence. Analysis of the BVH-3, BVH-11 and BVH-11-2 protein sequences suggested the presence of putative hydrophobic leader sequences.

5 \* encoded amino acids for the chimeras are expressed as the gene product, additional non essential amino acids residue were added M is methionine, K is lysine, L is leucine, G is glycine and P is proline.

10 Monoclonal antibody (Mab)-secreting hybridomas were obtained by fusions of spleen cells from immunized mice and non-secreting, HGPRT-deficient mouse myeloma SP2/0 cells by the methods of Fazekas De St-Groth and Scheidegger (J Immunol Methods 35 : 1-21, 1980) with modifications (J. Hamel et al. J Med Microbiol 23 : 163-170, 1987). Female BALB/c mice (Charles River, St-Constant, Quebec, Canada) were immunized with either BVH-3M (thioredoxin-His•Tag-BVH-3M fusion protein/ pET32 system), BVH-11M (thioredoxin-His•Tag-BVH-11M fusion protein/ pET32 system), BVH-11-2M (thioredoxin-His•Tag-BVH-11-2M fusion protein/ pET32 system), BVH-11B (thioredoxin-His•Tag-BVH-11B fusion protein/ pET32 system), BVH-3M (His•Tag-BVH-3 fusion protein/ pURV22.HIS system) or NEW1 (NEW1-His•Tag fusion protein/ pET21 system) gene products from S. pneumoniae strain SP64 to generate the Mab series H3-, H11-, H112-, H11B-, H3V-, and HN1-, respectively.

25 Culture supernatants of hybridomas were initially screened by enzyme-linked-immunoassay (ELISA) according to the procedure described by Hamel et al. (Supra) using plates coated with preparations of purified recombinant BVH-3, BVH-11 and/or BVH-11-2 proteins or suspensions of heat-killed S. pneumoniae cells.

30 The Mab-secreting hybridomas selected for further characterization are listed in Table 3 and Table 4 from the following Example 2. The class and subclass of Mab



immunoglobulins were determined by ELISA using commercially available reagents (Southern Biotechnology Associates, Birmingham, AL).

5 Furthermore, the cloning and expression of chimeric gene(s) encoding for chimeric polypeptides and the protection observed after vaccination with these chimeric polypeptides are described.

10 BVH-3 and BVH-11 gene fragments corresponding to the 3' end of the genes were amplified by PCR using pairs of oligonucleotides engineered to amplify gene fragments to be included in the chimeric genes. The primers used had a restriction endonuclease site at the 5' end, thereby allowing directional in-frame cloning of the amplified product into digested plasmid vectors (Table 1 and Table 2). PCR-amplified products were digested with restriction endonucleases and ligated to linearized plasmid pET21 or pSL301 vector. The resultant plasmid constructs were confirmed by nucleotide sequence analysis. The recombinant pET21 plasmids containing a PCR product were linearized by digestion with restriction enzymes for the in-frame cloning of a second DNA fragment and the generation of a chimeric gene encoding for a chimeric pneumococcal protein molecule. Recombinant pSL301 plasmids containing a PCR product were digested with restriction enzymes for the obtention of the DNA inserts. The resulting insert DNA fragments were purified and inserts corresponding to a given chimeric gene were ligated into pET21 vector for the generation of a chimeric gene. The recombinant chimeric polypeptides listed in Table 2 were as C-terminal fusion with an His-tag. The expressed recombinant proteins were purified from supernatant fractions obtained from centrifugation of sonicated IPTG-induced E. coli cultures using a His-Bind metal chelation resin (QIAGEN, Chatsworth, CA).

Groups of 8 female BALB/c mice (Charles River) were immunized subcutaneously two times at three-week intervals with 25  $\mu$ g of either affinity purified His•Tag-fusion protein identified in

5 presence of 15-20  $\mu$ g of QuilA adjuvant. Ten to 14 days following the last immunization, the mice were challenged challenged intravenously

with 10E5-10E6 CFU of *S. pneumoniae* type 3 strain WU2. The polypeptides and fragments are capable of eliciting a protective

10 immune response.

**Table 2A**

Experiment	Immunogen	Alive : Dead	Days to death post-infection
1	none	0 : 8	1, 1, 1, 1, 1, 1, 1, 1
	NEW 1	2 : 6	1, 2, 2, 2, 2, 2, >14, >14
	NEW 13	1 : 7	1, 1, 3, 3, 4, 5, 5, >14
	NEW 12	6 : 2	3, 11, 6X >14
	BVH-3M	1 : 7	3, 3, 3, 3, 3, 3, 3, >14
2	none	0 : 8	1, 1, 1, 1, 1, 1, 1, 1
	NEW 17	7 : 1	4, 7 X >14
	NEW 12	3 : 5	3, 3, 3, 4, 5, >14, >14, >14
3	none	0 : 8	2, 2, 2, 2, 2, 2, 2, 2
	NEW 18	1 : 7	2, 2, 2, 2, 3, 3, 3, 3
	NEW 19	8 : 0	8 X >14
	NEW 10	8 : 0	8 X >14
	BVH-11-2	8 : 0	8 X >14

15

## EXAMPLE 2

This example describes the identification of peptide domains carrying target epitopes using Mabs and recombinant truncated proteins described in example 1.

Hybridomas were tested by ELISA against truncated BVH-3, BVH-11 or BVH-11-2 gene products in order to characterize the epitopes recognized by the Mabs. The truncated gene products were generated from S. pneumoniae SP64 strain except for BVH-3-Sp63 which was generated from S. pneumoniae SP63 strain. As a positive control, the reactivity of each antibody was examined with full-length BVH-3, BVH-11 or BVH-11-2 recombinant proteins. In some cases, the Mab reactivity was evaluated by Western immunoblotting after separation of the gene product by SDS-PAGE and transfer on nitrocellulose paper. The reactivities observed is set forth in the following Table 3 and Table 4.

Table 3. ELISA reactivity of BVH-3-reactive Mabs with a panel of eleven BVH-3 gene products and the BVH-11M molecule

Gene products tested												
Mabs (IgG isotype)	BVH- 3M	BVH- 3AD	BVH- 3B	BVH- 3C	NEW 1	NEW 2	NEW 3	NEW 21	NEW 22	NEW 23	BVH- 3 sp63	BVH- 11M
H3-4F9 (1)	+	+	-	+	-	-	-	-	-	-	+	+
H3-4D4 (1)	+	+	-	+	-	-	-	-	-	-	+	+
H3-9H12 (1)	+	+	-	+	-	-	-	-	-	-	+	+
H3-7G2 (1)	+	+	-	-	-	-	-	-	+	-	-	-
H3-10A1 (1)	+	+	-	-	-	-	-	+	-	+	+	-
H3-4D3 (1)	+	-	+	-	+	-	+	+	-	-	+	-
H11-6E7 (1)	+	+	-	+	-	-	-	NT	NT	NT	+	+
H11-10H10 (2a)	+	+	-	+	-	-	-	NT	NT	NT	+	+
H11-7G11 (2b)	+	+	+	+	+	+	-	NT	NT	NT	+	+
H3V-4F3 (1)	+	-	+	-	+	-	-	+	-	-	+	-
H3V-2F2 (1)	+	-	+	-	+	+	-	+	-	-	+	-
H3V-7F4 (1)	+	-	+	-	+	+	-	+	-	-	+	-
H3V-7H3 (1)	+	-	+	-	+	-	+	+	-	-	+	-

Gene products tested												
Mabs (IgG isotype)	BVH- 3M	BVH- 3AD	BVH- 3B	BVH- 3C	NEW 1	NEW 2	NEW 3	NEW 21	NEW 22	NEW 23	BVH- 3 sp63	BVH- 11M
H3V-13B8 (1)	+	-	+	-	+	-	+	+	-	-	+	-
H3V-9C2 (1)	+	+	-	+/-	-	-	-	-	+	-	+/-	+/-
H3V-9C6 (1)	+	+	-	-	-	-	-	-	+	-	-	-
H3V-16A7 (1)	+	+	-	-	-	-	-	+	-	+	-	-
H3V-15A10 (1)	+	+	+	+/-	+	+	-	+	+	+	+	+/-
H3V-6B3 (1/2)	+	+	NT	NT	+	+	-	+	+	-	NT	-
HN1-5H3 (2b)	+	-	+	NT	+	-	-	+	-	-	+	-
HN1-8E3 (2a)	+	-	+	NT	+	-	-	+	-	-	+	-
HN1-14F6 (2a)	+	-	+	NT	+	-	-	+	-	-	+	-
HN1-2G2 (1)	+	-	+	NT	+	+	-	+	-	-	+	-

Gene products tested												
Mabs (IgG isotype)	BVH- 3M	BVH- 3AD	BVH- 3B	BVH- 3C	NEW 1	NEW 2	NEW 3	NEW 21	NEW 22	NEW 23	BVH- 3 Sp63	BVH- 11M
HN1-12D8 (2a)	+	-	+	NT	+	+	-	+	-	-	+	-
HN1-14B2 (2a)	+	-	+	NT	+	+	-	+	-	-	+	-
HN1-1G2 (2a)	+	-	+	NT	+	-	+	+	-	-	+	-
HN1-10C12 (1)	+	-	+	NT	+	-	+	+	-	-	+	-
HN1-3E5 (1)	+	+	-	-	+	+	-	+	-	+	+	-

NT : not tested

+/- : very low reactivity but higher than background, possible non-specific Mab binding  
 Table 4. ELISA reactivity of BVH-11 and/or BVH-11-2-reactive Mabs with a panel of  
 fourteen BVH-11 and BVH-11-2 gene products and the BVH-3M molecule

Gene products tested																
Mabs (IgG isotype)	BVH- 11M	BVH- 11A	BVH- 11B	BVH- 11C	NEW 5	NEW 6	NEW 7	NEW 8	NEW 9	NEW 10	NEW 11	NEW 14	NEW 18	NEW 19	BVH- 11- 2-M	BVH- 3M
H3-4F9 (1)	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
H3-4D4 (1)	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
H3-9H12 (1)	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
H11-6E7 (1)	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
H11- 10H10 (2a)	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
H11-7G11 (2b)	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
H11-1B12 (1)	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
H11-7B9	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-

Gene products tested																
Mabs (IgG isotype)	BVH- 11M	BVH- 11A	BVH- 11B	BVH- 11C	NEW 5	NEW 6	NEW 7	NEW 8	NEW 9	NEW 10	NEW 11	NEW 14	NEW 18	NEW 19	BVH- 11- 2-M	BVH- 3M
(2a)																
H11-3H5 (1)	+	-	+	+	+	-	-	-*	-	+	-	+	+	-	+	-
H11-10B8 (1)	+	-	+	+	+	-	-	-*	-	+	-	+	+	-	+	-
H11-1A2 (1)	+	-	+	+	+	-	-	-*	-	+	-	+	+	-	+	-
H112-3A1 (1)	+	-	+	NT	+	-	-	+	-	+	-	+	+	-	+	-
H112- 13C11 (1)	+	+/-	+	NT	+	-	-	+	-	+	-	+	+	-	+	-
H112- 10H10 (1)	+	+	-	NT	+	-	-	+	-	+	-	+	+	-	+	-
H112-1D8 (2a)	+	+	-	NT	+	-	-	+	-	+	-	+	+	-	+	-



Gene products tested																
Mabs (IgG isotype)	BVH- 11M	BVH- 11A	BVH- 11B	BVH- 11C	NEW 5	NEW 6	NEW 7	NEW 8	NEW 9	NEW 10	NEW 11	NEW 14	NEW 18	NEW 19	BVH- 11- 2-M	BVH- 11- 3M
H112- 10G9 (2b)	+	-	+	NT	+	-	-	-	+	+	-	+	-	+	+	-
H112- 10A2 (1)	+	-	+	NT	+	-	-	+/-	+	+	-	+	-	+	+	-
H112-3E8 (2a)	+	-	+	NT	+	-	-	+/-	-	+	-	+	-	+	+	-
H112- 10D7 (2a)	+	-	+	NT	+	-	-	-	-	+	-	+	-	-	+	-
H112-2H7 (2a)	+	+	-	NT	-	-	-	-	-	-	-	-	-	-	+	-
H112-6H7 (1)	+	+	-	NT	-	-	-	-	-	-	-	-	-	-	+	-
H112-3A4 (2a)	-	-	-	NT	-	-	-	-	-	+	+	-	-	+	+	-
H112-	-	-	-	NT	-	-	-	-	-	+	+	-	-	+	+	-



Gene products tested																
Mabs	BVH- 11M	BVH- 11A	BVH- 11B	BVH- 11C	NEW 5	NEW 6	NEW 7	NEW 8	NEW 9	NEW 10	NEW 11	NEW 14	NEW 18	NEW 19	BVH- 11- 2-M	BVH- 3M
(IgG isotype)																
13D5 (2)																
H11B- 11B8 (1)	+	-	+	+	+	-	-	-	+	+	-	+	-	+	+	-
H11B- 7E11 (1)	+	-	+	+	+	-	-	-	-	+	-	+	-	-	+	-
H11B-1C9 (1)	+	-	+	+	+	-	-	-	-	+	-	+	-	-	+	-
H11B-5E3 (2)	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-
H11B-6E8 (1)	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-

NT : not tested

+/- : very low reactivity but higher than background, possible non-specific Mab binding

\* : a strong signal was detected when tested by Western immunoblotting

The deduced locations of the epitopes are summarized in Figure 28 and Figure 29. As can be seen from the data in Table 3, BVH-3-reactive Mabs can be divided into two groups : BVH-3A- and BVH-3B-reactive Mabs with the exception of Mabs H11-7G11 and H3V-15A10 which reacted with both, BVH-3A and BVH-3B molecules. The BVH-3A-reactive Mabs can be subdivided in two subgroups of antibodies depending of their reactivity or lack of reactivity with BVH-3C recombinant protein. Mab reactive with BVH-3C protein recognized epitopes shared by both, BVH-3 and BVH-11 proteins. As can be seen in Table 4, these BVH-3- and BVH-11-cross-reactive Mabs were also reactive with BVH-11A and BVH-11-2M recombinant proteins. BVH-3B-reactive Mabs can be subdivided into three subgroups according to their reactivity with NEW1, NEW2 and NEW3 recombinant proteins. Some Mabs were only reactive with the NEW1 protein while other Mabs were reactive with either, NEW1 and NEW2 or NEW1 and NEW3 recombinant proteins.

Mabs H11-7G11 and H3V-15A10 react with epitopes in more than one position on BVH-3. The reactivity of H11-7G11 with BVH-3AD, BVH-3B, BVH-3C, BVH-11A and BVH-11-2M molecules suggests that H11-7G11 epitope might comprised HXXHXXH sequence. This sequence is repeated, respectively, 6 and 5 times in BVH-3 and BVH-11/BVH-11-2 protein sequences. The lack of reactivity of Mab H11-7G11 with NEW 10 molecule suggests that the epitope includes the HGDHXXH sequence. Multiple-position mapping of H3V-15A10 epitope on BVH-3 is suggested by the reactivity of the Mab with two BVH-3 fragments that do not overlap.

Interestingly, Mabs H3-7G2, H3V-9C6 and H3V-16A7 were not reactive with BVH-3 Sp63 thus allowing the location of their corresponding epitopes on a 177-amino acid fragment comprised

between amino acids 244 and 420 on BVH-3 molecule of S. pneumoniae SP64 (Figure 31).

As can be seen from the data in Table 4, the Mabs that are reactive with BVH-11- and/or BVH-11-2 and that do not recognize BVH-3 molecules can be divided into three groups according to their reactivities with BVH-11A and NEW10 recombinant proteins. Some Mabs reacted exclusively with either BVH-11A or NEW10 protein while other Mabs were reactive with both, BVH-11A and NEW10 recombinant proteins.

### EXAMPLE 3

This example describes the construction of BVH-3 and BVH-11-2 gene libraries for the mapping of epitopes.

BVH-3 and BVH-11-2 gene libraries were constructed using recombinant pCMV-GH and PSL301 plasmid DNA containing respectively, BVH-3 gene sequence spanning nucleotides 1837 to 4909 (SEQ ID NO: 2) or BVH-11-2 gene sequence spanning nucleotides 172 to 2630 (SEQ ID NO: 5) and the Novatope® library construction and screening system (Novagen). The recombinant plasmids containing BVH-3 or BVH-11-2 gene fragment were purified using QIAGEN kit (Chatsworth, CA) and digested with the restriction enzymes BglII and XbaI respectively. The resulting BglII-XbaI DNA fragments were purified using the QIAquick gel extraction kit from QIAGEN and digested with Dnase I for the generation of randomly cleaved DNA. DNA fragments of 50 to 200 bp were purified, treated with T4 DNA polymerase to blunt the target DNA ends and add a single 3'dA residue, and ligated into pSCREEN-T-Vector (Novagen) following the procedures suggested by the manufacturer (Novatope® System, Novagen). The gene libraries of E. coli clones, each of which expressing a small

peptide derived from BVH-3 or BVH-11-2 genes were screened by standard colony lift methods using Mabs as immunoprobcs. The colony screening was not successful with Mabs producing very high backgrounds on colony lifts. Moreover, in some cases, Mabs failed to detect epitope-expressing-colonies. The lack of reactivity can possibly be explained by the small amount of recombinant proteins produced or the recognition of conformation-dependent epitopes consisting of different protein domains. Sequencing of DNA inserts from positive clones determined the location of the segment that encodes the target epitope. The data are presented in Table 5. The peptides encoded by DNA inserts into the recombinant pSCREEN-T vector can be purified and used as immunogens as described below in Example 6.

The peptide sequences obtained from the screening of BVH-3 and BVH-11-2 gene libraries with the Mabs are in agreement with the Mab ELISA reactivities against the truncated gene products. As expected, the amino acid sequences obtained from H11-7G11 contained the sequence HGDHXXH. These findings provide additional evidence for the location of epitopes recognized with the Mabs. Interestingly, although the Mabs H112-10G9, H112-10A2 and H11B-11B8 were reactive against the same peptide sequence (amino acid residues 594 to 679 on BVH-11-2 protein sequence), clones corresponding to the sequence spanning from amino acid residues 658 to 698 were only picked up by Mab H11B-11B8 thus revealing the location of H11B-11B8 epitope between amino acid residues 658 to 679 (SEQ ID NO: 163). Mabs H112-10G9, H112-10A2, and H11B-11B8 are directed against 3 distinct non overlapping epitopes located closely on the peptide sequence corresponding to amino acid residues 594 to 679 (SEQ ID NO: 22).

Table 5. Peptide sequences obtained from the screening of BVH-3 and BVH-11-2 gene libraries with Mabs

Mab	Clone/ Protein designat ion	Nucleotide position	Amino acid position	Amino acid sequence	SEQ ID NO
H3-4D4	4D4.9	SEQ ID 1: 226-509	SEQ ID 6: 76-169	DQGYVTSHGDDHYHYNGKVPYDALFSEELMKDPNYQLKDA DIVNEVKGGYIIKVDGKYVYVYLKDAAHADNVRTKDEINRQK QEHVKDNEKVNS	11
H11- 7G11	7G11.7	SEQ ID 1: 193-316	SEQ ID 6: 64-105	GIOAEQIVIKITDQGYVTSHGDDHYHYNGKVPYDALFSEEL L	12
H11- 7G11	7G11.9	SEQ ID 1: 1171-1284	SEQ ID 6: 390-428	TAYIVRHGDHFFHYIPKSNQIGQPTLPNNSLATPSPSLPI	13
H3-4D3	4D3.4	SEQ ID 1: 2565-2670	SEQ ID 6: 855-890	TSNSTLEEVPVTPVQEKVAKFAESYGMKLENVLFN	14
HN1- 8E3	8E3.1	SEQ ID 1: 3004-3120	SEQ ID 6: 1016-1039	MDGTIELRLPSGEVIKKNLSDFIA	15
HN1- 1G2	1G2.2	SEQ ID 1: 3017-3120	SEQ ID 6: 1005-1039	YGLGLDSVIFNMDGTIELRLPSGEVIKKNLSDFIA	16
HN1- 10C12	10C12.7	SEQ ID 1: 2936-3120	SEQ ID 6: 983-1039	PALEEAPAVDPVQEKLEKFTASYGLGLDSVIFNMDGTIELR LPSGEVIKKNLSDFIA	17
HN1- 14F6	14F6.3	SEQ ID 1: 2501-2618	SEQ ID 6: 833-872	KVEEPTSEKVEKEKLSETGNSTSNSTLEEVPVTPVQEK	18

# Table 3: Sequences

Mab	Clone/ Protein designat ion	Nucleotide position	Amino acid position	Amino acid sequence	SEQ ID NO
HN1- 12D8	B12D8.2	SEQ ID 1:1433- 1767	SEQ ID 6: 512-589	MKDLKKIEEKIAGIMKQYGVKRESIVVNKEKNAILIYPHGD HHHADPIDEHKPVGIGHSHSNYELFKPEEGVAKKEGN	19
H3V- 7F4	7F4.1	SEQ ID 1: 1633-1785	SEQ ID 6: 545-595	AIYYPHGDHHHADPIDEHKPVGIGHSHSNYELFKPEEGVAK KEGNKVTGTE	20
H112- 10D7	10D7.5	SEQ ID 5: 1685-1765	SEQ ID 8: 525-553	IQVAKLAGKYTTEDGYIFDPRDITSDEGD	21
H112- 10G9	10G9.3	SEQ ID 5: 1893-2150	SEQ ID 8: 594-679	DHQDSGNTAKGAEEIYNRVKAAKKVPLDRMPYNLQYTVEV KNGSLIIPHYDHYHNIKFEWFDEGLYEAPKGSLEDLLATV KYYV	22
H112- 10A2	10A2.2	SEQ ID 5: 1893-2150	SEQ ID 8: 594-679	DHQDSGNTAKGAEEIYNRVKAAKKVPLDRMPYNLQYTVEV KNGSLIIPHYDHYHNIKFEWFDEGLYEAPKGSLEDLLATV KYYV	22
H11B- 11B8	B11B8.1	SEQ ID 5: 1893-2150	SEQ ID 8: 594-679	DHQDSGNTAKGAEEIYNRVKAAKKVPLDRMPYNLQYTVEV KNGSLIIPHYDHYHNIKFEWFDEGLYEAPKGSLEDLLATV KYYV	22
H11B- 11B8	11B8.4	SEQ ID 5: 2085-2217	SEQ ID 8: 658-698	GLYEAPKGSLEDLLATVKYYVEHPNERPHSDNCGFNASDH	23
H112- 3A4.1	3A4.1	SEQ ID 5: 2085-2217	SEQ ID 8: 658-698	VENSVINAKIADAEEALLEKVTDPsirQNAMEtTLtGLKSSLL	24



Mab	Clone/ Protein designat ion	Nucleotide position	Amino acid position	Amino acid sequence	SEQ ID NO
3A4		2421-2626	769-837	LGTKDNNTISAEVDSLALLKESQPAPI	

#### EXAMPLE 4

This example describes the immunization of animals with recombinant proteins for the generation of antibody reactive with BVH-3, BVH-11 and/or BVH-11-2.

NZW rabbits (Charles River Laboratories, St-Constant, Québec, Canada) were immunized subcutaneously at multiple sites with 50 µg or 100 µg of the purified BVH-3M, L-BVH-3AD, NEW1, NEW13, or L-BVH-11 recombinant protein in presence of 80 µg of QuilA adjuvant (Cedarlane Laboratories Ltd, Hornby, Canada). The rabbits were boosted two times at three-week intervals with the same antigen and blood samples were collected before each immunization and 6 to 28 days following the last immunization. The sera samples were designated preimmune, post 1<sup>st</sup>, post 2<sup>nd</sup> or post 3<sup>rd</sup> injection. The rabbit immune response to immunization was evaluated by ELISA using recombinant BVH-3M (BVH-3M-His•Tag fusion protein/ pET21 system) or BVH-11M (BVH-11M-His•Tag fusion protein/ pET21 system) proteins or suspensions of heat-killed S. pneumoniae Rx-1 cells as coating antigens. ELISA titer was defined as the reciprocal of the highest sera dilution at which absorbance A<sub>410</sub> value was 0.1 above the background value. Antibodies reactive with BVH-3 and/or BVH-11 epitopes were elicited following immunization in all animals as shown in the following Table 6. Antibody reactive with recombinant or pneumococcal antigens was not present in the preimmune sera. The immune response to immunization was detectable in the sera of each rabbit after a single injection of recombinant antigen. The antibody response following the second injection with either antigen tested was characterized by a strong increase in antibody titer. Interestingly, good titers of antibody reactive with S. pneumoniae cells, with an average titer of 52,000 after the third immunization, were obtained, thus establishing that

native pneumococcal epitopes are expressed on the recombinant E. coli gene products. These data support the potential use of BVH-3, BVH-11 and/or BVH-11-2 gene products and the antibody raised to BVH-3, BVH-11 and/or BVH-11-2 gene products as

5 vaccines for the prevention and the treatment of pneumococcal disease, respectively.

Table 6. Rabbit Antibody response to immunization with BVH-3 and BVH-11 gene products

Rabbit	Immunogen	Sera sample	ELISA Titer with coating antigen		
			BVH-3M	BVH-11M	<u>S. pneumoniae</u>
#15	BVH-3M (50µg)	Preimmune	NT	NT	200
		Post-1 <sup>st</sup>	NT	NT	1,600
		Post-2 <sup>nd</sup>	NT	NT	20,000
		Post-3 <sup>rd</sup>	512,000	NT	40,000
#16	BVH-3M (100µg)	Preimmune	NT	NT	200
		post 1 <sup>st</sup>	NT	NT	1,600
		post 2 <sup>nd</sup>	NT	NT	40,000
		post 3 <sup>rd</sup>	10 <sup>6</sup>	NT	80,000
#112	L-BVH-3AD (50 µg)	Preimmune	<100	NT	NT
		post 1 <sup>st</sup>	16,000	NT	NT
		post 2 <sup>nd</sup>	512,000	NT	NT
		post 3 <sup>rd</sup>	2x10 <sup>6</sup>	NT	32,000
#113	New 1 (50 µg)	Preimmune	<100	NT	NT
		post 1 <sup>st</sup>	16,000	NT	NT
		post 2 <sup>nd</sup>	512,000	NT	NT
		post 3 <sup>rd</sup>	10 <sup>6</sup>	NT	64,000
#114	New 13	Preimmune	NT	<100	NT
		post 1 <sup>st</sup>	NT	16,000	NT

	(50 $\mu$ g)	post 2 <sup>nd</sup>	NT	64,000	NT
		post 3 <sup>rd</sup>	NT	256,000	32,000
#116	L-BVH-11 (50 $\mu$ g)	Preimmune	NT	<100	NT
		post 1 <sup>st</sup>	NT	64,000	NT
		post 2 <sup>nd</sup>	NT	10 <sup>6</sup>	NT
		post 3 <sup>rd</sup>	NT	2x10 <sup>6</sup>	64,000

NT : not tested

#### EXAMPLE 5

This example describes the protection of animals against fatal experimental pneumococcal infection by administration of antibody raised to BVH-3, BVH-11 or BVH-11-2 gene products.

High-titer Mab preparations were obtained from ascites fluid of mice inoculated intraperitoneally with Mab-secreting hybridoma cells according to the method described by Brodeur et al (J Immunol Methods 71 :265-272, 1984). Sera samples were collected from rabbits immunized with BVH-3M as described in Example 4. The rabbit sera collected after the third immunization and ascites fluid were used for the purification of antibodies by precipitation using 45 to 50% saturated ammonium sulfate. The antibody preparations were dissolved and dialyzed against phosphate-buffered saline (PBS).

CBA/N (xid) mice (National Cancer Institute, Frederick, MA) were injected intraperitoneally with either 0.1 ml of purified rabbit antibodies or 0.2 ml of ascites fluid before intravenous challenge with approximately 200 CFU of the type 3 S. pneumoniae strain WU2. Control mice received sterile PBS or antibodies

purified from preimmune rabbit sera or sera from rabbits immunized with an unrelated N. meningitidis recombinant protein antigen. One group of mice was challenged with S. pneumoniae before the administration of anti-BVH-3 antibody. Samples of the S. pneumoniae challenge inoculum were plated on chocolate agar plates to determine the number of CFU and verify the challenge dose. The CBA/N mice were chosen because of their high susceptibility to S. pneumoniae infection. The LD<sub>50</sub> of WU2 injected intravenously to CBA/N mice is estimated to be ≤10 CFU. Deaths were recorded at 24-h intervals for a period of at least 7 days.

The protection data obtained from mice injected with rabbit anti-BVH-3 antibody are set forth in the following Table 7. Nine out of 10 mice receiving the anti-BVH-3 antibody survived the challenge in contrast to none of 10 mice injected with control antibody or PBS buffer. The observation that antibody raised to the BVH-3-M molecule passively protected even when administered after the challenge demonstrated the ability of anti-BVH-3 antibody to prevent death even from an already established infection.

Table 7. Protective effects of rabbit antibody to BVH-3-M gene in CBA/N mice challenged i.v. with WU2 pneumococci

Antibody preparation	Time of antibody administration	Alive : Dead	Days to death post-infection
Anti-BVH3M	1 h before infection	5 : 0	>14, >14, >14, >14, >14
Anti-N. meningitidis	1 h before infection	0 : 5	2, 2, 2, 2, 2
Anti-BVH-3M	0.5 h post-infection	4 : 1	2, >14, >14, >14, >14

None (PBS)	1 h before infection	0 : 5	1, 2, 2, 2, 2
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CBA/N mice were infected with 1000 CFU of WU2 S. pneumoniae before or after intraperitoneal administration of 0.1 ml of rabbit antibody.

- 5 In an other experiment, 0.1 ml of rabbit antibody prepared from preimmune and immune sera were administered intraperitoneally to CBA/N mice four hours before intranasal challenge with 280 CFU of S. pneumoniae P4241 type 3 strain. As seen in the following Table 8, all immunized mice survived the challenge while none of  
10 9 mice receiving preimmune sera antibody or buffer alone were alive on day 6 post-infection. S. pneumoniae hemocultures on day 11 post-challenge were negative for all surviving mice. Furthermore, 100% protection was observed in mice receiving monoclonal antibodies H112-10G9 or a mixture of H112-10G9 and H11B-7E11 which are directed against BVH-11/BVH-11-2.

Table 8. Protective effects of passive transfer of rabbit antibody to BVH-3-M gene product or anti-BVH-11/BVH-11-2 specific Mabs in CBA/N mice challenged i.n. with P4241  
20 pneumococci

Antibody preparation	Alive : Dead	Days to death post-infection
Anti-BVH-3M	5 : 0	>11, >11, >11, >11, >11
Antibody from preimmune sera	0 : 5	3, 3, 3, 6, 6
H112-10G9	4 : 0	>11, >11, >11, >11
H112-10G9+H11B- 7E11	5 : 0	>11, >11, >11, >11, >11

None (PBS)	0 : 4	3, 3, 3, 3
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Altogether, the results from Table 7 and Table 8 clearly establish that immunization of animals with a BVH-3 gene product such as BVH-3M elicited protective antibodies capable of preventing experimental bacteremia and pneumonia infections.

The protection data obtained for mice injected with ascites fluid are set forth in the following Table 9. Administration of a volume of 0.2 ml of ascites fluid of 0.2 ml of some sets of ascites fluid prevented death from experimental infection. For example, H112-3A4 + H112-10G9 and H112-10G2 + H112-10D7 sets of 2 Mabs conferred complete protection against experimental infection. These data indicated that antibody targetting BVH-11 and/or BVH-11-2 epitopes gave efficient protection. The Mabs H112-3A4, H112-10G9, H112-10D7, H112-10A2, H112-3E8, H112-10C5, H11B-11B8, H11B-15G2, H11B-1C9, H11B-7E11, H11B-13D5 and H11-10B8 were present in at least one protective pair of Mabs and were said to be protective and reactive against protective epitopes. The locations of protection-conferring epitopes on BVH-11-2 molecules are summarized in Table 10 and Figure 29 . Protective Mabs H112-3A4, H112-10G9, H112-10D7, H112-10A2, H112-3E8, H112-10C5, H11B-11B8, H11B-15G2, H11B-1C9, H11B-7E11, H11B-13D5 and H11-10B8 were all reactive with New 10 protein corresponding to amino acid residues 271 to 838 on the BVH-11-2 molecule. Six out of these 12 Mabs were directed against epitopes present in the NEW 19 protein and 3 protective Mabs recognized NEW 14. Interestingly, Mab H112-3A4 and H112-10C5 reacted with distinct epitopes exclusive to BVH-11-2 located at the carboxyl end comprised between amino acid residues 769 and 837. Also, Mabs H11-7G11, H11-6E7 and H3-4F9 reactive with epitopes shared by pneumococcal BVH-3, BVH-11 and BVH-11-2

molecules did not succeed to protect even if given in combination with protective H112-10G9 or H112-11B8 Mab. These Mabs recognized epitopes located at the amino end of the BVH-3, BVH-11 and BVH-11-2 molecules comprising, respectively, the first 225, 228 and 226 amino acid residues. The comparison of the BVH-3, BVH-11 and BVH-11-2 protein sequences revealed that a large number of amino acids were conserved in the amino end portion comprising these 225-228 residues with a global 72.8 % identity (Figure 32).

Altogether the data set forth in Table 9 and Table 10 suggest that the protection eliciting BVH-11- and BVH-11-2-epitopes is comprised in the carboxy terminal product containing amino acids 229 to 840 and 227 to 838, on BVH-11 and BVH-11-2 proteins, respectively.



Table 9. Passive immunization with BVH-11- and/or BVH-11-2-specific Mabs can protect mice from lethal experimental pneumococcal infection.

Experiment	Mab	Alive : Dead	Days to death post-infection
1	H112 3A4 + H112-10G9	6 : 0	6 X >10
	H112-3A4 + H112-10D7	5 : 1	4, 5X >10
	None	0 : 6	2, 2, 2, 2, 2, 6
2	H112-10 A2 + H112-10D7	5 : 1	3, 5X >10
	H112-3E8 + H112-10G9	6 : 0	6 X >10
	None	0 : 6	2, 2, 2, 2, 2, 2
3	H112-10D7 + H11B-11B8	6 : 0	6 X >10
	H112-10G9 + H11B-15G2	3 : 3	2, 6, 6, 3 X >10
	None	0 : 6	2, 2, 2, 2, 2, 2
4	H112-10G9 + H112-10D7	5 : 0	5 X >11
	None	0 : 5	2, 2, 2, 2, 2
5	H112-10G9 + H11-10B8	4 : 1	8, 4 X >14
	H112-10G9 + H11B-7E11	5 : 0	5 X >14
	None	0 : 3	1, 2, 2
6	H112-10G9 + H11B-1C9	4 : 1	4, 4 X >14
	None	0 : 3	2, 2, 2
7	H112-10C5 + H11B-13D5	5 : 0	5 X >14
	None	3 : 3	2, 2, 2

- 5 CBA/N mice were injected intraperitoneally with a total of 0.2 ml of ascites fluid 4 hours before intravenous challenge with S. pneumoniae WU2.

Table 10. Deduced locations of protection-conferring epitopes on BVH-11-2 molecules.

Mabs	Protection	Gene products carrying Mab-epitope
H112-3A4	+	NEW 19 and NEW 11
H112-10G9	+	NEW 19
H112-10D7	+	NEW 14 and NEW 10
H112-10A2	+	NEW 19
H112-3E8	+	NEW 19
H11B-11B8	+	NEW 19
H11B-15G2	+	NEW 18
H11B-7E11	+	NEW 14 and NEW 10
H11-10B8	+	NEW 18
H11B-1C9	+	NEW 14 and NEW 10
H112-3A1	-	NEW 18 and NEW 8
H112-10H10	-	NEW 18 and NEW 8
H112-2H7	-	BVH-11-2M
H112-6H7	-	BVH-11-2M
H11-7G11	-	BVH-11A and BVH-3C
H11-6E7	-	BVH-11A and BVH-3C
H112-10C5	+	NEW 19, NEW11 and 3A4.1
H11B-13D5	+	NEW 19
H112-7G2	-	NEW 18
H112-7E8	-	BVH-11-2M
H3-4F9	-	BVH-11A and BVH-3C

- 5 Altogether the data presented in this example substantiate the potential use of antibodies raised to BVH-3, BVH-11 or BVH-11-2 molecules as therapeutic means to prevent, diagnose or treat S. pneumoniae diseases.

## EXAMPLE 6

This example describes the localization of surface-exposed peptide domains using Mabs described in Example 1.

5

S. pneumoniae type 3 strain WU2 was grown in Todd Hewitt (TH) broth (Difco Laboratories, Detroit MI) enriched with 0.5% Yeast extract (Difco Laboratories) at 37°C in a 8% CO<sub>2</sub> atmosphere to give an OD<sub>600</sub> of 0.260 (~10<sup>8</sup> CFU/ml). The bacterial suspension was then aliquoted in 1 ml samples and the S. pneumoniae cells were pelleted by centrifugation and resuspended in hybridoma culture supernatants. The bacterial suspensions were then incubated for 2 h at 4°C. Samples were washed twice in blocking buffer [PBS containing 2% bovine serum albumin (BSA)], and then 1 ml of goat fluorescein (FITC)-conjugated anti-mouse IgG + IgM diluted in blocking buffer was added. After an additional incubation of 60 min at room temperature, samples were washed twice in blocking buffer and fixed with 0.25 % formaldehyde in PBS buffer for 18-24 h at 4°C. Cells were washed once in PBS buffer and resuspended in 500 µl of PBS buffer. Cells were kept in the dark at 4°C until analyzed by flow cytometry (Epics® XL; Beckman Coulter, Inc.). Ten thousands (10,000) cells were analyzed per sample and the results were expressed as % Fluorescence and Fluorescence index (FI) values. The % Fluorescence is the number of fluorescein-labelled S. pneumoniae cells divided by 100 and the FI value is the median fluorescence value of pneumococci treated with Mab supernatant divided by the fluorescence value of pneumococci treated with the conjugate alone or with a control unrelated Mab. A FI value of 1 indicated that the Mab has not been detected at the surface of the bacteria whereas a FI value higher than 2 was considered positive when at least 10 % of the pneumococcal cells were labelled and indicated that the Mab was reactive with cell-

surface exposed epitopes. The following Table 11 summarized the data obtained with the Mabs tested by flow cytometry.

Flow cytometric analysis revealed that the Mabs reactive with BVH-3C and/or BVH-11A molecules did not bind to the cell surface. In contrast, with the exception of Mabs H3V-9C6 and H3V-16A7, the Mabs reactive with NEW 1, NEW 2, NEW 3, NEW 22 or NEW 23 BVH-3 gene products were detected at the surface of pneumococci. These data indicated that the first 225 amino acid residues located at the amino end of BVH-3 are internal. The lack of binding of Mabs H3V-9C6 and H3V-16A7 suggest some portions of the sequence corresponding to the 177-amino acids absent from the BVH-3 molecule of S. pneumoniae SP63 appears not to be accessible to antibodies.

Results from BVH-11- and/or BVH-11-2-reactive Mabs revealed that there is a good correlation between surface-exposure and protection. All Mabs reactive with internal epitopes as determined by the flow cytometry assay were not protective whereas all the protective Mabs described in Example 5 gave a positive signal in flow cytometry. Although an FI value of 9.0 and a % Fluorescence of 81.2 were obtained with Mab H11-7G11, this Mab was not shown to protect. Additional assays can be used to further evaluate whether this Mab and its corresponding epitope might participate in anti-infectious immunity.

Table 11. Results from the binding of Mabs at the surface of S. pneumoniae by flow cytometry analysis

Mab	% Fluorescence	FI	Binding	Gene products carrying Mab-epitope
H3-4F9	3.4	1.2	-	BVH-3C and BVH-11A
H3-4D4	3.4	1.2	-	BVH-3C and BVH-11A

Mab	% Fluorescence	FI	Binding	Gene products carrying Mab-epitope
H3-9H12	2.5	1.1	-	BVH-3C and BVH-11A
H3-7G2	66.2	6.3	+	NEW 22
H3-10A1	58.8	5.6	+	NEW 23
H3-4D3	33.2	3.5	+	NEW 3
H3V-4F3	24.4	2.9	+	NEW 1
H3V-2F2	15.6	2.4	+	NEW 2
H3V-7F4	58.7	5.6	+	NEW 2
H3V-7H3	68.8	6.9	+	NEW 3
H3V-13B8	75.0	7.7	+	NEW 3
H3V-9C2	66.4	6.2	+	NEW 22
H3V-9C6	2.9	1.0	-	NEW 22
H3V-16A7	6.6	1.7	-	NEW 23
H3V-15A10	58.7	5.7	+	NEW 22 and NEW 23
HN1-5H3	43.4	5.3	+	NEW 1
HN1-8E3	57.4	6.6	+	NEW 1
HN1-14F6	57.8	6.7	+	NEW 1
HN1-2G2	54.8	6.3	+	NEW 2
HN1-12D8	14.3	3.0	+	NEW 2
HN1-14B2	11.5	2.7	+	NEW 2
HN1-1G2	59.9	7.0	+	NEW 3
HN1-10C12	13.6	2.8	+	NEW 3
H11-6E7	4.9	1.2	-	BVH-3C and BVH-11A
H11-10H10	6.5	1.6	-	BVH-3C and BVH-11A
H11-7G11	81.2	9.0	+	BVH-3C and NEW 2

Mab	% Fluorescence	FI	Binding	Gene products carrying Mab-epitope
H11-1B12	3.1	1.2	-	BVH-11A
H11-7B9	2.4	1.1	-	BVH-11A
H11-10B8	81.1	9.1	+	NEW 18 and NEW 8
H11-1A2	84.4	10	+	NEW 18 and NEW 8
H11-3H5	84.0	9.8	+	NEW 18 and NEW 8
H112-13C11	49.3	5.9	+	NEW 18 and NEW 8
H112-10H10	0.4	1.0	-	BVH-11A and NEW 18
H112-1D8	0.4	1.0	-	BVH-11A and NEW 18
H112-10G9	78.9	10.4	+	NEW 19
H112-10A2	75.5	9.6	+	NEW 19
H112-3E8	62.5	7.5	+	NEW 19
H112-10D7	64.5	7.7	+	NEW 14
H112-2H7	0.7	1.1	-	BVH-11A
H112-6H7	0.3	1.0	-	BVH-11A
H112-3A4	70.1	8.9	+	NEW 11
H112-10C5	86.3	9.2	+	NEW 11 AND 3A4.1
H112-14H6	89.6	11	+	NEW 11
H112-14H6	0.8	1.4	-	NEW 11
H112-7G2	4.7	2.0	-	NEW 18
H112-13H10	0.5	1.0	-	NEW 18

Mab	% Fluorescence	FI	Binding	Gene products carrying Mab-epitope
H112-7E8	0.4	1.0	-	BVH-11-2M
H112-7H6	0.2	1.0	-	BVH-11-2M
H11B-5F10	3.1	1.1	-	NEW 18
H11B-15G2	60.2	5.7	+	NEW 18 and NEW 8
H11B-13D5	75.7	8.3	+	NEW 19
H11B-11B8	78.4	8.3	+	NEW 19
H11B-7E11	32.3	3.5	+	NEW 14
H11B-1C9	57.3	5.5	+	NEW 14
H11B-5E3	1.8	1.0	-	NEW 7
H11B-6E8	2.4	1.0	-	NEW 7

#### EXAMPLE 7

5 This example describes the immunization of animals with peptide epitopes of BVH-3 and BVH-11-2.

The recombinant pSCREEN-T vector (Novagen, Madison, WI) containing DNA fragment (nucleotides 2421 to 2626 on SEQ ID NO :  
10 5) encoding the Mab 3A4-epitope (SEQ ID NO: 24) was transformed by electroporation (Gene Pulser II apparatus, BIO-RAD Labs, Mississauga, Canada) into E. coli Tuner ( $\lambda$ DE3) pLysS [BL21 (F' ompT hsdSB (rB<sup>-</sup>mB<sup>-</sup>) gal dcm lacYI pLysS (Cm<sup>r</sup>)] (Novagen). In  
15 this strain, the expression of the fusion protein is controlled by the T7 promoter which is recognized by the T7 RNA polymerase

(present on the  $\lambda$ DE3 prophage, itself under the control of the lac promoter inducible by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). The pLysS plasmid reduces the basal fusion protein expression level by coding for a T7 lysozyme, which is a natural inhibitor of the T7 RNA polymerase.

The transformants were grown at 37°C with 250 RPM agitation in LB broth (peptone 10g/l, yeast extract 5g/l, NaCl 5g/l) supplemented with 50mM glucose, 100 $\mu$ g/ml carbenicillin and 34 $\mu$ g/ml chloramphenicol, until the absorbance at 600nm reached a value of 0,7. The overexpression of T7gene 10 protein-His•Tag-3A4.1 fusion protein was then induced by the addition of IPTG to a final concentration of 1mM and further incubation at 25°C with 250 RPM agitation for 3 hours. Induced cells from a 800-ml culture were pelleted by centrifugation and frozen at -70°C. The fusion protein was purified from the soluble cell fraction by affinity chromatography based on the binding of a six histidine residues sequence (His-Tag) to divalent cations ( $\text{Ni}^{2+}$ ) immobilized on a metal chelation Ni-NTA resin (Qiagen, Mississauga, Canada). Briefly, the pelleted cells were thawed and resuspended in Tris buffered sucrose solution (50mM Tris, 25%(w/v) sucrose) and frozen at -70°C for 15 minutes. Cells were incubated 15 minutes on ice in the presence of 2mg/ml lysozyme before disruption by sonication. The lysate was centrifuged at 12000 RPM for 30 minutes and Nickel charged Ni-NTA resin (QIAGEN) was added to the supernatant for an overnight incubation at 4°C, with 100 RPM agitation. After washing the resin with a buffer consisting of 20mM Tris, 500mM NaCl, 20mM imidazole pH 7,9, the fusion 3A4.1 protein was eluted with the same buffer supplemented with 250mM imidazole. The removal of the salt and imidazole was done by dialysis against PBS at 4°C.



The protein concentration was determined with BCA protein assay reagent kit (Perce, Rockford, IL) and adjusted to 760 µg/ml.

To evaluate whether immunization with an epitope peptide sequence could confer protection against disease, groups of 6 female CBA/N (xid) mice (National Cancer Institute) are immunized subcutaneously three times at three-week intervals with affinity purified T7gene10 protein-His•Tag-3A4.1 fusion protein or, as control, with QuilA adjuvant alone in PBS. Twelve to fourteen days following the third immunization, the mice are challenged intravenously with S. pneumoniae WU2 strain or intranasally with P4241 strain. Samples of the S. pneumoniae challenge inoculum are plated on chocolate agar plates to determine the number of CFU and to verify the challenge dose. The challenge dose are approximalety 300 CFU. Deaths are recorded daily for a period of 14 days and on day 14 post-challenge, the surviving mice are sacrificed and blood samples tested for the presence of S. pneumoniae organisms. The 3A4.1 protein or other tested protein is said protective when the number of mice surviving the infection or the median number of days to death is significantly greater in the 3A4.1-immunized group compared to the control mock-immunized group.

#### EXAMPLE 8

This example illustrates the improvement of the antibody response to pneumococci using BVH-3 fragments and variants thereof.

The combined results obtained from studies of Mab reactivity with truncated gene products, epitope-expressing colonies and live intact pneumococci presented in examples 2, 3 and 6, allowed to delineate between surface-exposed and internal

epitopes. The epitopes detected by Mabs that efficiently bound to pneumococci cells mapped to a region comprised between amino acid residues 223 to 1039 of BVH-3 described in SEQ ID NO 6. The existence of protective epitopes in the BVH-3-carboxyl half was confirmed by demonstrating that mice immunized with NEW1 molecule were protected from fatal infection with P4241 strain.

Gene sequence comparison revealed that in some strains, the region of BVH-3 encoding for amino acids 244 to 420 as described in SEQ ID NO6 is absent thus suggesting the lack of utility of this sequence in vaccine to prevent disease caused by such strains (SEQ ID NO: 9 versus SEQ ID NO: 1). Further BVH-3 fragments or variants thereof were designed in the purpose to develop a universal highly effective vaccine that would target the immune response to ubiquitous surface-exposed protective epitopes. BVH-3 gene fragments designated NEW1 (encoding amino acid residues 472 to 1039 from SEQ ID NO: 6) and NEW40 (encoding amino acid residues 408 to 1039 from SEQ ID NO: 6) were amplified from the S. pneumoniae strain SP64 by PCR using pairs of oligonucleotides engineered for the amplification of the appropriate gene fragment. Each of the primers had a restriction endonuclease site at the 5'end, thereby allowing directional in-frame cloning of the amplified product into the digested plasmid vector. PCR-amplified products were digested with restriction endonucleases and ligated to linearized plasmid pET21 (Novagen) expression vector digested likewise.

Oligonucleotide	primers	HAMJ489
(ccgaattccatatgcaaattgggcaaccgactc;	NdeI)	and HAMJ279
(cgccaagcttcgctatgaaatcagataaattc;	HindIII)	were used for the

NEW 40 construction. Clones were first stabilized in E. coli DH5 $\alpha$  before introduction into E. coli BL21 ( $\lambda$ DE3) for expression of the truncated gene products. Variants from NEW1 and NEW40 were generated by mutagenesis using the Quickchange Site-

Directed Mutagenesis kit from Stratagene and the oligonucleotides designed to incorporate the appropriate mutation. The presence of 6 histidine tag residues on the C-terminus of the recombinant molecules simplified the purification of the proteins by nickel chromatography. The following tables 12 and 13 describe the sequences of the primers used for the mutagenesis experiments and the variant gene products generated, respectively. Mutagenesis experiments using primer sets 39, 40, 46, 47 or 48 resulted in silent changes and were performed in the purpose of improving the expression of the desired gene or gene fragment since it was observed that during the course of expression, BVH-3 gene and fragments of, shorter secondary translation initiation products were coexpressed.

Table 12. List of PCR oligonucleotide primer sets used for site-directed mutagenesis on BVH-3 gene truncates

Primer set	Primer identification	SEQ ID No	Primer SEQUENCE 5' ---> 3'
9	HAMJ513	177	GAATCAGGTTTGTTCATGAGTTCCGGAGACCACAAATCATTTATTC
	HAMJ514	178	GAAATAATGATTGTGGTCTCCGGAACATCATGACAAAACCTGATTC
10	HAMJ515	179	GTCATGAGTTCCGGAGACTCCAAATCATTTATTTCTTCAAGAAGG
	HAMJ516	180	CCTTCTTGAAGAAATAATGATTGGAGTCTCCGGAACATCATGAC
11	HAMJ517	181	ATGAGTTCCGGAGACTCCAAATTTCTTATTTCTTCAAGAAGGACTTG
	HAMJ518	182	CAAGTCCTTCTTGAAGAAATAAGAAATTGGAGTCTCCGGAACATCAT
14	CHAN51	183	GCGATTATTTATCCGTCCTGGAGATCACCATCATGC
	CHAN52	184	GCATGATGGTGATCTCCAGACGGATAAATAATCGC
17	CHAN53	185	CCGTCCTGGAGATGCCATCATGCAGATCCG
	CHAN54	186	CGGATCTGCATGATGGCCATCTCCAGACGG
19	CHAN47	187	CCGACGGAGATAAGCGTCATGCAGATCCGATTG
	CHAN48	188	CAATCGGATCTGCATGACGCTTATCTCCCTGCGG
20	CHAN55	189	CCGTCCTGGAGATGGCACTCATGCAGATCCGATTG
	CHAN56	190	CAATCGGATCTGCATGAGTGCCATCTCCAGACGG
22	CHAN57	191	CCGTCCTGGAGATGGCACTTCTGCAGATCCGATTGATG
	CHAN58	192	CATCAATCGGATCTGCAGAAAGTGCCATCTCCAGACGG
23	HAMJ523	193	CCGCATGGAGATGGCCATCATGCAGATCCG
	HAMJ524	194	CGGATCTGCATGATGGCCATCTCCATGCGG
24	HAMJ526	195	GTCATGAGTCACGGAGACTCCAATCATTTCTTCAAGAAGG
	HAMJ527	196	CCTTCTTGAAGAAATAATGATTGGAGTCTCCGTGACTCATGAC
25	HAMJ528	197	ATGAGTCACGGAGACCACAATTCTTATTTCTTCAAGAAGGACTTG
	HAMJ529	198	CAAGTCCTTCTTGAAGAAATAAGAAATTGTGGTCTCCGTGACTCAT
29	HAMJ569	199	TACCTCATTAATGACTCTTACTCTAACATCAAAATTTGAGTGGTTTG
	HAMJ570	200	CAACCACTCAAAATTTGATGTTAGAGTAAGAGTCATAATGAGGTA
30	HAMJ571	201	TACCTTCTTATGACCATTAATCTCTAACATCAAAATTTGAGTGGTTTG
	HAMJ572	202	AAACCACTCAAAATTTGATGTTAGAGTAATGGTCATAAGAAGGTA
31	HAMJ573	203	AACGGTAGTTTAAATCATACCTTCTTAAAGACCATTACCATAACATC
	HAMJ574	204	GATGTTATGGTAATGGTCTTTTAGAAGGTATGATTAACTACCGTT
32	HAMJ575	205	CGGTAGTTTAAATCATACCTCATAAAGGACTCTTACCATAACATCAAAA

Primer set	Primer identification	SEQ ID No	Primer SEQUENCE 5' ---> 3'
33	HAMJ576	206	TTTGATGTTATGGTAAGAGTCCTTATGAGGTATGATTAAACTACCG
	HAMJ577	207	AACGGTAGTTTAATCATACCTGACCATTAACCATAAACATCAAAATTG
	HAMJ578	208	CAAAATTGATGTTATGGTAATGGTCAGGTATGATTAAACTACCGTT
34	HAMJ579	209	AACGGTAGTTTAATCATACCTTAACCATAAACATCAAAATTGAGTGG
	HAMJ580	210	CCACTCAAAATTGATGTTATGGTAAGGTATGATTAAACTACCGTT
35	HAMJ581	211	ACCGTAGTTTAATCATACCTTAACCATCAAAATTGAGTGGTTTGAC
	HAMJ582	212	GTCAAACCACTCAAAATTGATGTTAGGTATGATTAAACTACCGTT
37	HAMJ536	213	CCTATGTAACCTCCACATATAACCCATAGCCACTGG
	HAMJ537	214	CCAGTGGCTATGGGTTATATGTGGAGTTACATAGG
39	HAMJ550	215	CGTGAAGATATTGTCGTAAATAAAGAAAAAATGCCG
	HAMJ551	216	CGCAATTTTCTTTATTTACGACAATACTTTTCACG
40	HAMJ586	217	CATGAAGAAAGATGTTACGGTTTCGATGCTAACCGTATTATCGCTGAAG
	HAMJ587	218	CTTCAGCGATAAATACGGTTAGCATCGAAACCGTAACCATCTTCTCTG
41	HAMJ588	219	GAATCAGGTTTTGTCTCATGAGTGACCAACAATCATTTCTTC
	HAMJ589	220	GAAGAAATAATGATTGTGTGTCACCTCATGACAAAACCTGATTTC
42	HAMJ590	221	GAAGATGAATCAGGTTTTGTCTCATGAGTAAATCATTTCTTCAAG
	HAMJ591	222	CTTGAAGAAATAATGATTACTCATGACAAAACCTGATTCTCTTC
43	HAMJ592	223	GAAGATGAATCAGGTTTTGTCTCATGAGTTATTTCTTCAAGAGGAC
	HAMJ593	224	GTCCTTCTTGAAGAAATAAATCACTCATGACAAAACCTGATTCTCTTC
44	HAMJ594	225	AAAATGCGATTATTTATCCGCACCATCATGCGAGATCCGATTG
	HAMJ595	226	CAATCGGATCTGCAATGATGGTGGGATAAATAATCGCATTTT
45	HAMJ600	227	AAAATGCGATTATTTATCCGGCAGATCCGATTGATGAACATAAAC
	HAMJ601	228	GTTTATGTTTCATCAATCGGATCTGCCGGATAAAATAATCGCATTTT
46	HAMJ604	229	GATGCTAACCGTATAATCGCCGAAGACGAATCAGGTTTGTTCATG
	HAMJ605	230	CATGACAAAACCTGATTCTGCTTCCGGGATTATACGGTTAGCATC
47	HAMJ606	231	CGCCGAAGACGAATCCGGCTTTGTAAATGAGTCACGGAGACTCC
	HAMJ607	232	GGAGTCTCCGTGACTCATTAACAAGCCGGATTCTGTTCCGGCG
48	HAMJ608	233	CATCTCATGAACAGGATTATCCCGGCAACGCCAAAGAAATGAAAG
	HAMJ609	234	CTTTCATTTCTTTGGCGTTGCCGGGATAATCCTGTTTCATGAGATG

Table 13. Lists of truncated variant BVH-3 gene products generated from S. pneumoniae  
SP64

Protein designation	Gene/ Protein SEQ ID NO	Protein Identification*	PCR primer set (ref. table 12)	Gene used for mutagenesis
NEW1- mut1**	255	NEW1	39	NEW1
NEW35A	256	NEW1 550-SGDGTS-555	14, 17, 20, 22	NEW1
NEW42	257	NEW40 55-SGD <sup>-</sup> SN <sup>-</sup> S-60 144-SGDGTS-149	9, 10, 11, 14, 17, 20, 22	NEW40
NEW49	258	NEW40 55-SGDH <sup>-</sup> NH-60	9	NEW40
NEW50	259	NEW40 55-SGD <sup>-</sup> SNH-60	10	NEW49
NEW51	260	NEW40 55-SGDH <sup>-</sup> NH-60 144-SGDHHH-149	14	NEW49
NEW52	261	NEW40 55-SGD <sup>-</sup> SNH-60 144-SGDGHH-149	10, 17	NEW51
NEW53	262	NEW40 55-HGDH <sup>-</sup> NH-60 144-SGDHHH-149	14	NEW40
NEW54	263	NEW40 55-SGDH <sup>-</sup> NH-60 144-SGDGHH-149	17	NEW53
NEW55	264	NEW1 550-HGDGHH-555	23	NEW1
NEW56	265	NEW40 55-HGD <sup>-</sup> SNH-60 144-SGDHHH-149	24	NEW53
NEW56- mut2**	266	NEW56	40	NEW56
NEW56- mut3**	267	NEW56	46, 47, 48	NEW56
NEW57	268	NEW40 55-HGDH <sup>-</sup> NS-60 144-SGDHHH-149	25	NEW53
NEW63	269	NEW40 55-HGD <sup>-</sup> SNH-60 144-HGDHHH-149	24	NEW40
NEW64	270	NEW40 55-HGDH <sup>-</sup> NS-60 144-HGDHHH-149	25	NEW40
NEW65	271	NEW40 55-HGD <sup>-</sup> SNH-60 144-HGDGHH-149	23	NEW63

Protein designation	Gene/Protein SEQ ID NO	Protein Identification*	PCR primer set (ref. table 12)	Gene used for mutagenesis
NEW66	272	NEW40 55-HGDHNS-60 144-HGDGHH-149	23	NEW64
NEW76	273	NEW40 55-HGDHNS-60 144-SGDGHH-149	17	NEW64
NEW105	274	NEW40 55-____-60	41, 42, 43	NEW40
NEW106	275	NEW40 144-____-149	44, 45	NEW40
NEW107	276	NEW40 55-____-60 144-____-149	44, 45	NEW105

\* The underlined amino acid residues represent the modification in protein sequence. Nucleotides/amino acid residues are deleted in NEW105, NEW106 and NEW107 constructs.

\*\* silent mutation, i.e. the polypeptide is the same as New1.

Groups of 7 or 8 female BALB/c mice (Charles River) immunized as described earlier in example 1 were used for protection experiments against intranasal challenge with virulent S. pneumoniae P4241 strain. The mice were observed for 10 to 14 days post-infection. Data from Table 15 clearly indicate that the NEW35A molecule was equivalent to the parental NEW1 in term of protection. Interestingly, high survival rates where obtained for NEW40- and NEW56-immunized groups with 7 and 8 survivors out of 8 animals, respectively. Similarly, NEW25 comprising amino acid residues 233 to 1039 protected 7 out of 8 animals from lethal infection.

Table 14. Protection mediated by BVH-3 fragments or variants thereof in experimental pneumonia

Experiment	Immunogen	Alive : Dead	Days to death post-infection
1	Quil A	0 : 8	4, 4, 4, 4, 4, 4, 4, 4
	NEW 1	5 : 3	5, 7, 7, >14, >14, >14, >14, >14
	NEW 35A	5 : 2	9, 10, >14, >14, >14, >14, >14
	NEW 40	7 : 1	13, >14, >14, >14, >14, >14, >14, >14
	BVH-3M	4 : 4	7, 8, 10, 12, >14, >14, >14, >14
2	Quil A	0 : 8	3, 3, 4, 4, 4, 4, 4, 4
	NEW 52	4 : 4	7, 7, 8, 9, >10, >10, >10, >10
	NEW56	8 : 0	8 X >10
	NEW 40	7 : 1	6, >10, >10, >10, >10, >10, >10, >10
3	QuilA	0 : 8	3, 3, 4, 4, 4, 4, 4, 4
	NEW25	7 : 1	6, >13, >13, >13, >13, >13, >13, >13

Additionally, flow cytometry analyses of the binding capacity of the sera antibodies from the vaccinated animals revealed that



NEW40 and NEW56 antibodies labelled live intact pneumococci more efficiently than antibodies raised to BVH-3M (Table 15).

Table 15. Binding of mouse sera antibodies at the surface of S. pneumoniae type 3 strain WU2 as measured by flow cytometry.

Antisera	Fluorescence index			
	Experiment 1	Experiment 2	Experiment 3	Mean $\pm$ SE
BVH-3M	9.2	11.4	14.5	11.7 $\pm$ 1.5
NEW1	11.5	10.1	nd*	10.8 $\pm$ 0.7
NEW35A	14.3	12.9	nd	13.6 $\pm$ 0.7
NEW40	20.4	19.1	20.2	19.9 $\pm$ 0.4
NEW56	nd	16.7	20.2	18.5 $\pm$ 1.8
NEW52	nd	16.6	19.3	18.0 $\pm$ 1.4
Adjuvant alone	1.9	1.6	1.2	1.6 $\pm$ 0.2

\* nd: not done

Cytometry results are expressed as fluorescence index value where the fluorescence index is the median fluorescence value of pneumococci treated with test sera divided by the background fluorescence value of pneumococci treated with the fluorescein conjugate alone. In these flow cytometric assays, all sera were used at a dilution of 1 :50 and the sera from mice immunized with BVH-3C fragment or QuilA adjuvant alone gave a value similar to the background value.

Altogether the protection and pneumococci antibody binding data indicate that vaccination using NEW1 or NEW40 molecules and variants thereof, directs the immune response to conserved protective surface-exposed epitopes.

EXAMPLE 9

This example describes the cloning and expression of a chimeric deletant BVH-11-2 gene encoding for a chimeric polypeptide corresponding to BVH-11-2 conserved protective surface-exposed epitopes present in most if not all S. pneumoniae strains.

5

BVH-11-2 gene fragments corresponding to 4 gene regions, were amplified by PCR using pairs of oligonucleotides engineered to amplify fragments originating from SEQ ID NO :5 spanning nucleotides 1662 to 1742, 1806 to 2153, 2193 to 2414 and 2484 to 10 2627 from S. pneumoniae strain Sp64 BVH-11-2 gene.

The primers used, HAMJ490-491, HAMJ492-HAMJ493, HAMJ494-HAMJ495, HAMJ496-HAMJ354 had a restriction endonuclease site at the 5' end, thereby allowing directional in-frame cloning of the amplified product into the digested pET21b(+) plasmid vector (Table 16). PCR-amplified products were digested with restriction endonucleases and ligated to linearized plasmid pSL301 vector digested likewise except for the PCR-amplified fragment obtained with the primer pair HAMJ490-HAMJ491. The HAMJ490-HAMJ491 PCR-amplified product was purified from agarose gel using a QIAquick gel extraction kit from QIAGEN (Chatsworth, CA) and ligated into pGEM-T plasmid vector without any prior restriction endonuclease digestion. The resultant plasmid constructs were confirmed by nucleotide sequence analysis. The 25 recombinant plasmids containing each of the four were digested with restriction endonucleases corresponding with the 5' end of each primer pair used for the PCR-amplification. The fragments were purified from agarose gel like described earlier and were all ligated to linearized plasmid pET21b (+) digested with the 30 restriction enzymes NdeI and HindIII for the in-frame cloning of the four different regions of the BVH11-2 gene. Clones were first stabilized in E.coli DH5α before introduction into E.coli

BL21 ( $\lambda$ DE3) for expression of a chimeric pneumococcal protein molecule.

The resulting NEW43 gene sequence (SEQ ID No 257) is described  
5 in Figure 33.

The deduced amino acid sequence of NEW43 protein (SEQ ID No 258) is described in Figure 34.

WDC99 450728-1.055190.0044

Table 16. List of PCR oligonucleotide primers used to construct the NEW43 , VP43S and NEW86

Primer	SEQ ID NO	Sequence 5' - 3'	Nucleotide position	Restriction sites
HAMJ490	259	ccgaattccatatgcaaattacctacactgatgatg	SEQ ID 5 :1662-1683	NdeI
HAMJ491	260	ggactagtatcaaagatat aaccgtcttc	SEQ ID 5 :1742-1722	SpeI
HAMJ492	261	ggactagttggattaaaaa agatagtttgtctg	SEQ ID 5 :1806-1830	SpeI
HAMJ493	262	ttcccgcggttcgacatag tacttgacagtcg	SEQ ID 5 :2153-2131	SacII
HAMJ494	263	ttcccgcggaacgctagtg accatgttcg	SEQ ID 5 :2193-2212	SacII
HAMJ495	264	cggggtaccaggaatttca gcctcatctgtg	SEQ ID 5 :2414-2393	KpnI
HAMJ496	265	cccgggtaccctagtgattta gacaaaatgctatggag	SEQ ID 5 :2484-2510	KpnI
HAMJ 354	65	cgccaagcttctgtatagg agccggttgac	SEQ ID 5 :2627-2608	HindIII
HAMJ 583	266	ggatcccgaggaggtatgat taaactaccg	SEQ ID 5 :2039-2021	SmaI
HAMJ 584	267	catgcccgggaacatcaaa tttgagtggtttgac	SEQ ID 5 :2058-2081	SmaI
HAMJ 610	268	cttgatcgacatatgttgg caggcaagtacacaacag	SEQ ID 5 :1701-1722	NdeI

Table 17. List of truncated BVH-11-2 gene fragments generated from *S. pneumoniae* SP64 for the construction of NEW43

PCR-primer sets	Gene fragment designation	Corresponding amino acid residues on SEQ ID NO: 8	Cloning vector
HAMJ490-HAMJ491	NEW43a	517-543	pGEM-T
HAMJ492-HAMJ493	NEW43b	565-680	pSL301
HAMJ494-HAMJ495	NEW43c	694-767	pSL301
HAMJ496-HAMJ354	NEW43d	791-838	pSL301

Table 18. Properties of NEW86 and VP43S genes generated from NEW43 gene

PCR-primer sets	Gene/Protein designation	Identification
HAMJ610-HAMJ354	VP43S	NEW43 C'end corresponding to residues 15-272)
HAMJ490-HAMJ583 HAMJ584-HAMJ354	NEW86	NEW43 109-__PG__-114

10 NEW43-derived molecules designated VP43S and NEW86 were generated from gene amplification and cloning experiments using PCR primers described in Tables 16 and 18 and pET21 expression plasmid vector. Variants from NEW43 were generated by

15 from Stratagene and the oligonucleotides designed to incorporate the appropriate mutation. The presence of 6 histidine tag residues on the C-terminus of the recombinant molecules simplified the purification of the proteins by nickel chromatography. The following tables 19 and 20 describe the

sequences of the primers used for the mutagenesis experiments and the NEW43 variant gene products generated, respectively.

5

Table 19. List of PCR oligonucleotide primer sets used for site-directed mutagenesis on NEW43 gene

Primer set	Primer identification	SEQ ID NO	Primer SEQUENCE 5' ---> 3'
1	HAMJ 497 HAMJ 498	269 270	AACGGTAGTTTAATCATACCTTCTTATGACCATTACCATAACATC GATGTTATGGTAATGGTCATAAGAAGGTATGATTAACTACCGTT
2	HAMJ499 HAMJ500	271 272	AATCATACCTTCTTATGACTCTTACCATAACATCAAATTTGAGTG CACTCAAATTTGATGTTATGGTAAGAGTCATAAGAAGGTATGATT
3	HAMJ501 HAMJ502	273 274	TACCTTCTTATGACTCTTACTCTAACATCAAATTTGAGTGTTTG CAAACCACTCAAATTTGATGTTAGAGTAAGAGTCATAAGAAGGTA
26	HAMJ530 HAMJ531	275 276	AATCATACCTCATTATGACTCTTACCATAACATCAAATTTGAGTG CACTCAAATTTGATGTTATGGTAAGAGTCATAATGAGGTATGATT
27	HAMJ532 HAMJ533	277 278	TACCTCATTATGACCATTACTCTAACATCAAATTTGAGTGTTTG CAAACCACTCAAATTTGATGTTAGAGTAATGGTCATAATGAGGTA
29	HAMJ569 HAMJ570	279 280	TACCTCATTATGACTCTTACTCTAACATCAAATTTGAGTGTTTG CAAACCACTCAAATTTGATGTTAGAGTAAGAGTCATAATGAGGTA
30	HAMJ571 HAMJ572	281 282	TACCTTCTTATGACCATTACTCTAACATCAAATTTGAGTGTTTG AAACCACTCAAATTTGATGTTAGAGTAATGGTCATAAGAAGGTA
31	HAMJ573 HAMJ574	283 284	AACGGTAGTTTAATCATACCTTCTAAAGACCATTACCATAACATC GATGTTATGGTAATGGTCTTTAGAAGGTATGATTAACTACCGTT
32	HAMJ575 HAMJ576	285 286	CGGTAGTTTAATCATACCTCATAAGGACTCTTACCATAACATCAA TTTGATGTTATGGTAAGAGTCCTTATGAGGTATGATTAACTACCG
33	HAMJ577 HAMJ578	287 288	AACGGTAGTTTAATCATACCTGACCATTACCATAACATCAAATTTG CAAATTTGATGTTATGGTAATGGTCAGGTATGATTAACTACCGTT
34	HAMJ579 HAMJ580	289 290	AACGGTAGTTTAATCATACCTTACCATAACATCAAATTTGAGTGG CCACTCAAATTTGATGTTATGGTAAGGTATGATTAACTACCGTT
35	HAMJ581 HAMJ582	291 292	ACCGGTAGTTTAATCATACCTAACATCAAATTTGAGTGTTTGAC GTCAAACCACTCAAATTTGATGTTAGGTATGATTAACTACCGTT

Table 20. List of NEW43 variant gene products generated from S. pneumoniae SP64

Polypeptide designation	Polypeptide SEQ ID NO	Polypeptide identification*	PCR primer set (ref. table 22)	Gene used for mutagenesis
NEW60	293	NEW43 109- <u>SYD</u> HYH-114	1	NEW43
NEW61	294	NEW43 109-HYD <u>SY</u> H-114	26	NEW43
NEW62	295	NEW43 109-HYDHY <u>S</u> -114	27	NEW43
NEW80	296	NEW43 109- <u>SYD</u> <u>SY</u> H-114	2	NEW60
NEW81	297	NEW43 109- <u>SYD</u> <u>SY</u> <u>S</u> -114	3	NEW80
NEW82	298	NEW43 109-HYD <u>SY</u> <u>S</u> -114	29	NEW61
NEW83	299	NEW43 109- <u>SYD</u> HY <u>S</u> -114	30	NEW60
NEW84	300	NEW43 109- <u>SKD</u> HYH-114	31	NEW60
NEW85	301	NEW43 109-HK <u>D</u> <u>SY</u> H-114	32	NEW61
NEW88D1	302	NEW43 109-__ <u>D</u> HYH-114	33	NEW43
NEW88D2	303	NEW43 109-____ <u>Y</u> H-114	34	NEW88D1
NEW88	304	NEW43 109-_____-114	35	NEW88D2

\* The underlined amino acid residues represent the modification in protein sequence. Nucleotides/amino acid residues are deleted in NEW88D1, NEW88D2 and NEW88 constructs.

Groups of 7 or 8 female BALB/c mice (Charles River) immunized as described earlier in example 1 were used for protection experiments against intranasal challenge with virulent S. pneumoniae P4241 strain. Data from Table 21 clearly indicate that NEW 19, NEW43 and variants thereof provided protection against experimental pneumonia.

Table 21. Protection mediated by NEW19 and NEW43 fragments or variants thereof in experimental pneumonia

Experiment	Immunogen	Alive : Dead	Median day alive
1	Quil A	0 : 8	4, 4, 4, 4, 4, 4, 4, 5
	NEW 19	7 : 1	5, 7X >14
	NEW 43	8 : 0	8X >14
2	Quil A	0 : 8	4, 4, 4, 4, 4, 5, 5, 5
	NEW 43	7 : 1	8, 7X >14
	NEW 80	6 : 2	5, 6, 6 X >14
	NEW 83	6 : 2	8, 10, 6 X >14
3	Quil A	0 : 8	4, 4, 4, 4, 5, 5, 5, 5
	NEW 43	7 : 1	5, 7X >8
	NEW 88D1	5 : 3	5, 6, 6, 6 X >8
	NEW 88D2	5 : 3	6, 6, 6, 6 X >8
	NEW 88	7 : 1	6, 7X >8
3	Quil A	0 : 8	4, 4, 4, 5, 5, 5, 5, 6
	NEW 60	8 : 0	8 X >8
	NEW 84	8 : 0	8 X >8
	NEW 85	5 : 3	5, 7, 7, 5 X >8
	NEW 86	5 : 3	5, 6, 6, 5 X >8

5

#### EXAMPLE 10

This example describes the cloning and expression of chimeric genes encoding for a chimeric protein corresponding to the carboxy-terminal region of BVH-3 or variants thereof in fusion, at either the carboxyl end or the amino end, to NEW43 or variants thereof.



The chimeric genes comprising a BVH-3 truncate variant gene and a NEW43 or NEW43 variant gene have been designed following the procedure described in example 1. The polypeptides encoded by these chimeric genes are listed in the table 22. Briefly, gene fragments to be included in a chimeric gene were amplified by PCR using pairs of oligonucleotides engineered so that the primers had a restriction endonuclease site at the 5' end, thereby allowing directional in-frame cloning of the amplified product into digested plasmid vectors (Table 23 and Table 24). PCR-amplified products were digested with restriction endonucleases and ligated to linearized plasmid pSL301 vector. The resultant plasmid construct were confirmed by nucleotide sequence analysis. The recombinant pSL301 plasmids containing a PCR product were redigested with the same endonuclease restriction enzyme for the obtention of the DNA inserts. The resulting insert DNA fragments were purified and inserts corresponding to a given chimeric gene were ligated into pURV22-NdeI vector for the generation of a chimeric gene. The expressed recombinant proteins were purified from supernatant fractions obtained from centrifugation of sonicated heat-induced E. coli cultures using multiple chromatographic purification steps.

Table 22. List of polypeptides encoded by chimeric genes comprising a BVH-3 truncate variant gene and a NEW43 or NEW43 variant gene

Polypeptide designation	SEQ ID NO	Identification
VP 89	327	M-New56 -GP- New43*
VP 90	328	M-New43 -GP- New56

Polypeptide designation	SEQ ID NO	Identification
VP 91	329	M-New52 -GP- New43
VP 92	330	M-New43 -GP- New52
VP 93	331	M-New56 -GP- New60
VP 94	332	M-New60 -GP- New56
VP 108	333	M-New56 -GP- New88
VP109	334	M-New88 -GP- New56
VP 110	335	M-New60 -GP- New105
VP 111	336	M-New60 -GP- New107
VP112	337	M-New88 -GP- New105
VP113	338	M-New88 -GP- New107
VP114	339	M-New80-GP- New105
VP115	340	M-New80 -GP- New107
VP116	341	M-New83 -GP- New105
VP117	342	M-New83 -GP- New107
VP119	343	M-New43S- GP-New105
VP120	344	M-New43S- GP-New107
VP121	345	M-New80S- GP-New105
VP122	346	M-New80S- GP-New107
VP123	347	M-New88S- GP-New105
VP124	348	M-New88S- GP-New107

\* Encoded amino acids for the chimeras are expressed as the gene product, additional amino acid residues were added. M is methionine, G is glycine and P is proline.

155  
A 7  
Table 23. List of PCR oligonucleotide primer pairs designed for the generation of the chimeric genes encoding the polypeptides listed in Table 22.

Primer set	PCR-primer identification	Gene used for PCR amplification	Corresponding position of the gene fragment on
49	HAMJ490-HAMJ471	Variant New43	N-terminal
50	HAMJ564-HAMJ556	Variant New43	C-terminal
51	HAMJ489-HAMJ359	Variant New40	N-terminal
52	HAMJ559-HAMJ557	Variant New40	C-terminal
53	HAMJ610-HAMJ471	Variant New43S	N-terminal

Table 24. List of PCR oligonucleotide primers designed for the generation of the chimeric genes encoding the polypeptides listed in Table 22.

Primer	SEQ ID NO	Sequence 5' - 3'	Restriction site
HAMJ490	259	ccgaattccatatgcaaattaccta cactgatgatg	NdeI
HAMJ471	168	atatgggcccctgtataggagccgg ttgactttc	ApaI
HAMJ564	327	atatgggccccaaattacctacact gatgatgagattcagg	ApaI
HAMJ556	328	ataagaatgcggccgcctactgtat aggagccggttgactttc	NotI
HAMJ489	329	ccgaattccatatgcaaattgggca accgactc	NdeI
HAMJ359	173	tcccggggcccgcctatgaaatcaga taaattc	ApaI
HAMJ559	330	atatgggccccaaattgggcaaccg actc	ApaI
HAMJ354	65	cgccaagcttctgtataggagccgg ttgac	HindIII

Primer	SEQ ID NO	Sequence 5' - 3'	Restriction site
HAMJ610	268	cttgatcgacatatgttggcaggca agtacacaacag	NdeI
HAMJ557	331	ataagaatgcggccgcttacgctat gaaatcagataaattc	NotI
HAMJ279	35	cgccaagcttcgctatgaaatcaga taaattc	HindIII

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